THE INFLUENCE OF HLA CLASS II POLYMORPHISMS ON THE PROPERTIES OF BACTERIAL SUPERANTIGENS

Martin Llewelyn

Thesis submitted in fulfilment of the requirements

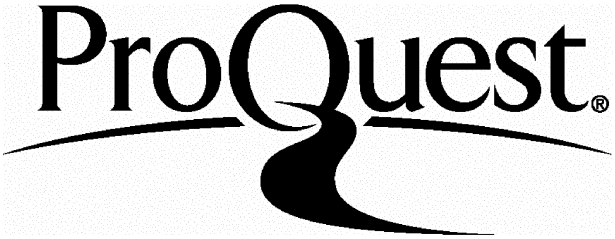
for the degree of PhD

Department of Infectious Diseases

Faculty of Medicine

Imperial College, London

This work was supported by the Medical Research Council (UK) through a Clinical Training Fellowship



ABSTRACT

The excessive and disordered immunological response triggered by bacterial superantigens has been implicated in the aetiology of a wide range of human diseases. The role of superantigens has been most clearly defined for the staphylococcal and streptococcal toxic shock syndromes. Since MHC class II presentation of superantigens to T cells is not MHC-restricted in the conventional antigen sense, the possibility that HLA polymorphisms could influence superantigenicity and thus, clinical susceptibility to the toxicity of individual superantigens, has received little attention. The work presented in this thesis focused principally on the streptococcal superantigen Streptococcal Pyrogenic Exotoxin A (SPEA) which utilises HLA-DQ but two staphylococcal superantigens, Staphylococcal Exotoxins A and B (SEA and SEB), were also studied.

Using HLA class II homozygous B lymphoblastoid cell lines and HLA-DQ transfectants of Bare Lymphocyte Syndrome cell lines, HLA-DQ polymorphisms were shown to markedly influence SPEA binding assessed by flow cytometry. Specifically, SPEA binding by cell lines expressing *HLA-DQA1*01* was up to ten fold higher than by cell lines expressing either *DQA1*03* or **05*. Using HLA-DQ molecules affinity purified from B lymphoblastoid cell lines, the impact of HLA-DQ polymorphisms on SPEA binding was confirmed in a cell free, ELISA based assay. Similar differences in SEA binding attributable to HLA-DR polymorphisms were observed in both the whole cell flow cytometry assay and the ELISA. Qualitative and quantitative differences were associated with differential HLA-DQ binding of SPEA

and HLA-DR binding of SEA. Using splenocytes from the murine Vβ8.2 transgenic mouse strain DO11.10 as responder cells, both proliferation and cytokine release in response to SPEA presented by *HLA-DQA1*01* were higher than when SPEA was presented by *HLA-DQA1*03* or **05*. Similar differences were observed using purified human T cells and using PBMCs from *HLA-DQA1* homozygous healthy donors. Quantitiative differences in T cell response to SEA presented by different DR alleles were also observed.

The Vβ specific changes in T cell repertoire that result from superantigen stimulation are regarded as being both the hall-mark of superantigenicity and defining of an individual superantigen. In the process of characterising the Vβ specific response to SPEA we noted that SPEA concentration has a profound effect on the repertoire of responding T cells. As the concentration of SPEA rises the T cell response broadens from Vβ14 to include Vβ12, 13.1 and 3. A similar effect was noted for SEA and SEB. Comparing the Vβ specific response in *DQA1* homozygous healthy donors differences in Vβ repertoire were found comparing *DQA1*01* donors with *DQA1*03* donors.

The work described in this thesis provides a molecular mechanism for observed differences in disease phenotype following infection by toxigenic strains of Staphylococci and Streptococci as well as the recent observation of an HLA association with predisposition to the severest manifestations of *S. pyogenes* infection. Given the widening perception of the importance of superantigens in disease pathogenesis, therapeutics and immunology the implications of these findings are very broad indeed. These data demonstrate that the HLA class II interaction with

bacterial superantigens is an important host – pathogen interface where host polymorphisms may have driven the generation of diversity in superantigen producing strains of bacteria much as HLA class II diversity has arisen largely in response to infectious disease.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Professor Jonathan Cohen and Dr Daniel Altmann for their guidance, support and encouragement throughout the course of this project. I am also greatly indebted to Dr Shiranee Sriskandan for her critical appraisal of my work. I am grateful also to those who collaborated with me in this work, especially Dr Giovanna Lombardi, of the Department of Immunology at Imperial College and Dr Mark Peakman of the Department of Immunology at Kings, Guys and St. Thomas' School of Medicine, also to Dr Daniel Douek of the Vaccine Research Center, National Institutes of Health, USA and Dr Bill Kwok of the Viginia Mason Research Center, Seattle, USA.

For my parents who showed me that knowledge is worth pursing and for Katy and Arran Llewelyn whose love and support have made this possible.

TABLE OF CONTENTS

FIGURES AND TABLES

Chapter 5

ABBREVIATIONS

APC	Antigen Presenting Cell
B-LCL	B Lymphoblastoid Cell Line
BLS	Bare Lymphocyte Syndrome
CDR	Complementarity Determining Region
CLA	Cutaneous Lymphocyte-associated Antigen
CLIP	Class II associated Invariant Chain Peptide
CPM	Counts per minute
DOC	Deoxycholic acid
FACS	Fluorescence activated cell sorting
FITC	Fluorescein Isothiocyanate
FR	Framework Region
GAS	Group A Streptococcus
HLA	Human Lymphocyte Antigen
Ii	Invariant Chain
IMGT	International ImMunoGeneTics project
MAM	Mycoplasma Arthritidis Mitogen
MBP	Myelin Basic Protein
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
Mls	Minor lymphocyte stimulating (antigen)
MLR	Mixed Lymphocyte Reaction
MMTV	Mouse Mammary Tumour Virus
MOG	Myelin Oligodendrocyte Glycoprotein
rSPEA	Recombinant SPEA
PAGE	Polyacrylamide Gel Electrophoresis
PE	Phycoerythin
PerCP	Peridinin chlorophyll
PLP	Proteolipid Protein

SAg	Superantigen
SEA, SEB etc	Staphylococcal Enterotoxin A, B etc
SDS	Sodium Dodecyl Sulphate
SMEZ	Streptococcal Mitogenic Exotoxin Z
SPEA	Streptococcal Pyrogenic Exotoxin A
STSS	Streptococcal Toxic Shock Syndrome
TCR	T cell receptor
TRBV	T cell receptor beta variable
TSST-1	Toxic Shock Syndrome Toxin - 1

CHAPTER 1

Introduction

1.1 Superantigens; an overview

1.1.1 General considerations

In 1989 Kappler and Marrack coined the term superantigen (White, Herman *et al.* 1989) and brought together lines of research which had been developing separately, in the fields of immunology and infectious diseases, for more than a decade. In the field of immunology Festenstein and co workers had described the minor lymphocyte stimulating (Mls) antigens of mice, which differentiated MHC identical mouse strains (Festenstein 1973), and these Mls antigens had been shown to stimulate T cells bearing particular T cell receptor (TCR) Vβ families (Abe, Ryan *et al.* 1987; Pullen, Marrack *et al.* 1988; Happ, Woodland *et al.* 1989). Mls antigens turned out to be encoded by the open reading frames of endogenous Mouse Mammary Tumour Virus proviruses (Acha-Orbea, Shakhov *et al.* 1991).

In the field of infectious diseases, the first bacterial superantigen was identified in the 1960s as a secreted toxin of *Staphylococcus aureus* and was named Staphylococcal enterotoxin A (SEA) for its potent enterotoxic properties (Chu, Thadhani *et al.* 1966). The mitogenic activity of SEA and the other pyrogenic exotoxins of *Staphylococcus aureus* and *Streptococcus pyogenes* was demonstrated much later (Barsumian, Schlievert *et al.* 1978; Schlievert and Gray 1989). The observation that these toxins appeared to stimulate T cells almost entirely via the Vβ region allowed Kappler and

Marrack to set out the common properties of superantigens for the first time (Marrack and Kappler 1990).

A wide diversity of superantigens is now recognised. The two best characterised groups remain the bacterial superantigens of *S. aureus* and *S. pyogenes*, and the superantigens of the exogenous and endogenous mouse mammary tumour viruses. Additional microbial superantigens of mycoplasma, enterobacteria and viruses have now been described, as have the first human endogenous retroviral superantigens (Cole, Knudtson *et al.* 1996; Huber, Hsu *et al.* 1996; Abe and Takeda 1997; Sutkowski, Conrad *et al.* 2001). The work described in this thesis concerns primarily the bacterial superantigens and the background given here will focus on this group.

The bacterial superantigens are a family of protein exotoxins which are; the Staphylococcal enterotoxins (SEs) A – K; Staphylococcal Toxic Shock Syndrome Toxin-1 (TSST-1); the Streptococcal Pyrogenic Exotoxins (SPEs) A, C and G-M; Streptococcal Superantigen (SSA) and Streptococcal Mitogenic Exotoxin Z (SMEZ). Particular structural and sequence features of these toxins result in the shared ability to bypass the mechanisms by which T cells are activated by conventional, MHC-restricted, peptide antigen. Conventional antigens are processed within antigen presenting cells (APCs) into peptide fragments which are loaded into the peptide binding groove of the MHC class II molecule for presentation at the cell surface to T cells (Shimonkevitz, Kappler *et al.* 1983). The T cell response is highly specific being restricted by the class II molecule, associated with CD4, and the specific peptide being presented. Thus only a tiny fraction of the host's T cell repertoire (<0.01%) will respond. In contrast, superantigens bind as intact proteins, directly to the MHC class

II molecule and to the T cell receptor extracellularly, at sites away from conventional peptide binding sites (Fraser 1989; Janeway, Yagi *et al.* 1989; Kappler, Kotzin *et al.* 1989; Choi, Herman *et al.* 1990). All bacterial superantigens bind the TCR at sites in the variable region of the β-chain (Vβ region). About 40 different Vβ regions are expressed in the human TCR. Most superantigens will bind several different Vβs stimulating all T cells expressing TCRs of those Vβ types (Choi, Kotzin *et al.* 1989; White, Herman *et al.* 1989). As a result, superantigens may trigger responses in 25-50% of resting T cells The contrasting feaures of superantigen and conventional peptide antigen presentation are illustrated in Fig. 1.1 (Llewelyn and Cohen 2002). The resulting excessive and uncoordinated release of inflammatory mediators, initially Tumour Necrosis Factor α (TNFα), followed by interleukin (IL)-6, interferon gamma (γ-IFN) and IL-2, is thought to be responsible for many of the features which result from exposure to these toxins (Miethke, Duschek *et al.* 1993).

1.1.2 Superantigen structure

Structural similarity among the bacterial superantigens is more marked than sequence homology. The crystal structures of SEA, SEB, SEC2, SED, TSST-1, SPEA and SPEC reveal a common structure comprising two globular domains linked by a long α-helix which runs down the length of the molecule. The C-terminal domain consists of a modified 'β-grasp motif' resembling that found in immunoglobulin binding domains and ubiquitin (Murzin, Brenner *et al.* 1995). The smaller N-terminal 'pseudo β-barrel' domain closely resembles the oligosaccharide/oligonucleotide-binding fold (OB) fold found in many bacterial toxins (Murzin 1993). Recombination of toxins with these different binding structures, which have become adapted to binding the

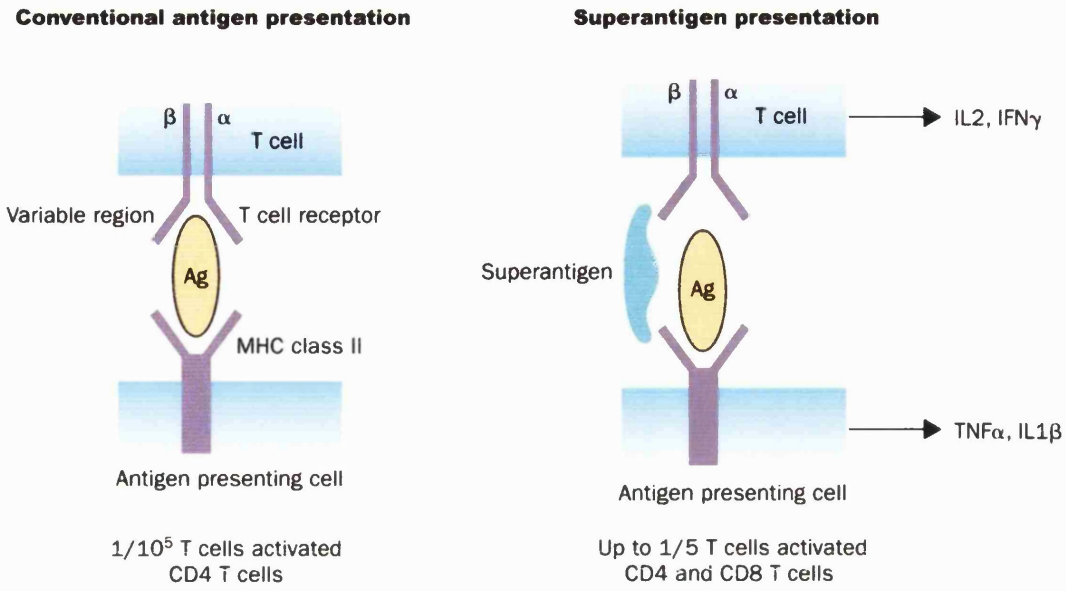


Fig. 1.1. Contrasting mechanisms of conventional antigen and superantigen presentation (Llewelyn and Cohen 2002).

MHC class II molecule and T cell receptor during microbial evolution, may be the origin of superantigens (Mitchell, Levitt *et al.* 2000). The degree of sequence homology which does exist between bacterial superantigens varies from as low as 15.5% (SEB to SEK) to 90% (SEA to SEE) but two highly conserved sequences have been identified (table 1.1) and are registered on the PROSITE database (Sigrist, Cerutti *et al.* 2002). Both lie in the core of the molecule away from sites involved in MHC class II or TCR interactions.

Table 1.1. Conserved amino acid sequences identified in bacterial superantigens

PROSITE designation	**Amino acid sequence**	
PS00278	K-X2-[LIVF]-X4-[LIVF]-D-X3-R-X2-L-X5-[LIV]-Y	All superantigens
PS00279	Y-G-G-[LIV]-T-X4-N	All except TSST-1 and SEH

The ribbon structure of SEB is shown in figure 1.2 to illustrate the general structural properties of superantigens and is discussed further below (Schad, Zaitseva *et al.* 1995).

The bacterial superantigens are not structurally related to other families of T cell superantigen.

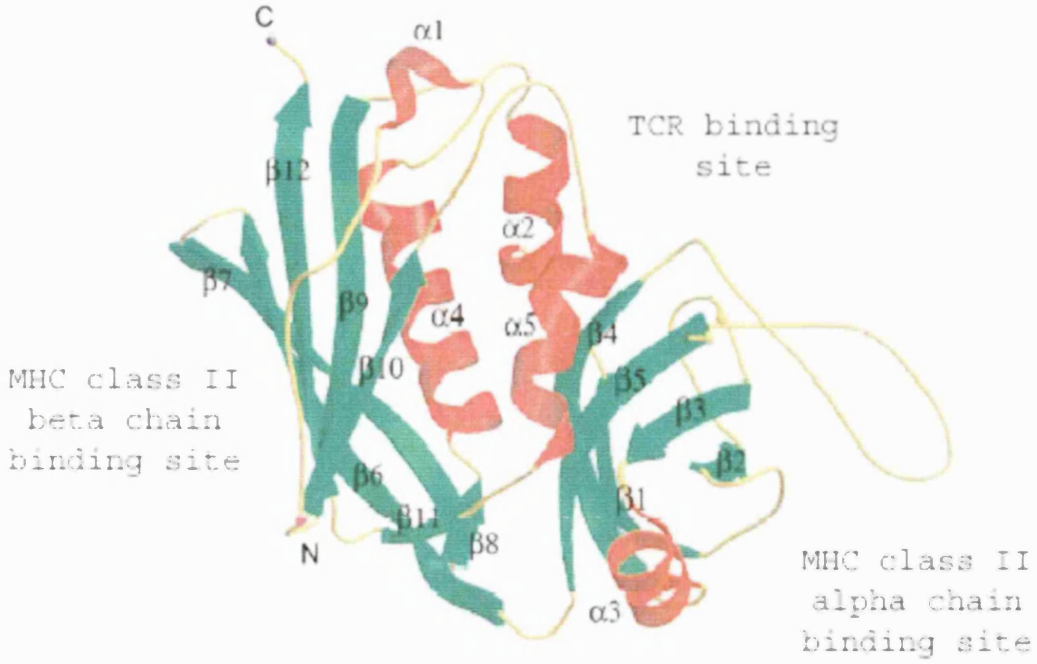


Fig. 1.2. Generic superantigen structural features. The figure has been modified from the structure of SEB (Schad, Zaitseva *et al.* 1995), which lacks the MHC class II β-chain binding site. Helices are coloured in red, β-sheets in green and loops in yellow. The regions of the superantigen molecules involved in binding to MHC class II and TCR are shown. Although SEB does not have an MHC class II β-chain binding site, the location of this site in superantigens which do use this binding mode is shown.

1.2 The class II Major Histocompatibility Complex

1.2.1 General considerations

MHC class II molecules, HLA class II in man, are cell surface, heterodimeric glycoproteins which function to present peptide antigen, derived from foreign and self proteins, to T cells. Three isotypes of cell surface HLA class II product exist, HLA-DP, DQ and DR each comprising an α and β chain coded for on chromosome 6 in the class II subregion of the HLA region. Two different expressed *HLA-DRB* genes may occur within the HLA region (Bontrop, Schreuder *et al.* 1986) allowing for expression of up to four HLA-DR molecules. Since two different HLA-DQ and DP molecules will be expressed in heterozygous individuals a total of eight class II molecules will be expressed on the surface of antigen presenting cells. Additionally, inter-isotypic mixed pairs may be expressed (Lotteau, Teyton *et al.* 1987). The HLA class II genes show extensive polymorphism. Most of the class II sequence polymorphism lies in the distal α1 and β1 domains at sites involved in interactions with peptide and the TCR. In addition to class II sequence polymorphism, differences in the regulation of class II exist such that marked differences in expression occur both between *HLA-DRB1* and *HLA-DQB1* and between different *HLA-DQB1* alleles (Gorga, Horejsi *et al.* 1987; Hume and Lee 1990; Andersen, Beaty *et al.* 1991). The level of DR expression is 5-25 times higher than the level of DQ expression in the peripheral immune system, and differences in tissue distribution and inducibility also occur (Reith, Barras *et al.* 1989; Andersen, Beaty *et al.* 1991; Douek and Altmann 2000).

The mouse homologues of HLA-DR (H-2E) and HLA-DQ (H-2A), are coded for by the genes *H-2E* and *H-2A* respectively. Many strains possess a mutation of the $H\text{-}2E_{\alpha}$ promotor which prevents expression of H-2E (Mathis, Benoist *et al.* 1983). Mice possess no functional gene equivalent of HLA-DP.

Although the interaction of superantigens with class II is thought to be an extracellular process, some aspects of the intracellular processing pathways involved in peptide association with class II are of importance in class II presentation of superantigen (Lavoie, Thibodeau *et al.* 1997). Peptide loaded into the class II binding groove is essential to the stability of the class II structure. During class II synthesis, prior to peptide loading, this stability is achieved in the endoplasmic reticulum by association with a monomorphic protein called invariant chain (Ii) (Cresswell 1994). Invariant chain is subsequently cleaved so that the class II molecule contains only class II invariant chain peptide (CLIP). CLIP is, in turn, replaced by antigenic peptide in a process which depends on a monomorphic class II molecule called HLA-DM. Unlike other class II molecules HLA-DM does not require peptide to stabilise its structure and is not expressed at the cell surface. It acts instead to shuttle peptide and CLIP in and out of the class II peptide binding cleft and to ensure that only stably bound peptide of the appropriate length (around 12 amino acids) and sequence are loaded into class II for expression at the cell surface (Cresswell 1994; Lanzavecchia 1996).

1.2.2 HLA class II nomenclature

Definition of the polymorphic products of the class II region began in the 1960s with serological typing using human antisera (van Rood 1993). Refinement of serotypes by application of HLA-specific monoclonal antibodies and subsequently by genetic typing, using sequencing and PCR, is an ongoing process. Currently 652 HLA class II alleles are recognised by the WHO nomenclature committee including 3 DR alpha, 418 DR beta, 24 DQ alpha and 55 DQ beta chain polymorphisms (Robinson, Waller *et al.* 2003). The accepted nomenclature relates to genotypes and no accepted nomenclature exists for the gene products. However, terms referring to serological subdivisions of HLA-DR and -DQ are widely used. Since serotyping relates essentially to β-chain differences, and the DR α-chain is non-polymorphic, the correlation between nomenclature for the *DRB1* gene locus, which codes the DR β-chain, and commonly used serological terms is fairly straightforward. The only complexity arises from the linkage which exists in certain DR types with *DRB3*, *DRB4* and *DRB5,* which code for DR52, DR53 and DR51 respectively. This results in expression of two DR molecules from any one HLA region in most class II haplotypes. A simplified list of DR alleles and serological equivalents of importance for this work is shown in table 1.2.

Table 1.2. Common HLA-DR types referred to in this study

Serogroup	Common Splits	Linkage with other *DRB* loci
DR1	*DRB1*01* = **DR1**	none
DR2	*DRB1*15* = **DR15** *DRB1*16* = **DR16**	linkage with DR51
DR3	*DRB1*03(01,04,05)* = **DR17** *DRB1*03(02,03)* = **DR18**	linkage with DR52
DR4	*DRB1*04* = **DR4**	linkage with DR53
DR5	*DRB1*11* = **DR11** *DRB1*1107* = **DR3/DR11** *DRB1*1117* = **DR14** *DRB1*12* = **DR12**	linkage with DR52
DR6	*DRB1*13* = **DR13** *DRB1*14* = **DR14**	linkage with DR52
DR7	*DRB1*07* = **DR7**	linkage with DR53
DR8	*DRB1*08* = **DR8**	none
DR9	*DRB1*09* = **DR9**	linkage with DR53
DR10	*DRB1*10* = **DR10**	none
DR51	*DRB5*01* = **DR51**	
DR52	*DRB3*01(02,03)* = **DR52**	
DR53	*DRB4*01* = **DR53**	

The situation for HLA-DQ is more complex since both α and β chains are highly polymorphic. The serogroups DQw1, 2, 3 and 4 were split such that DQw1 became DQ5 and DQ6, and DQw3 became DQ7 and DQ8. Table 1.3 correlates the common DQ serotypes and genotypes referred to in this work (Marsh, Parham *et al.* 2000). Much of the work in this thesis concerns the interaction of SPEA with the α-chains of DQ5, 6, 7, and 8. Since DQ5 and DQ6 comprise very similar α-chains, coded for by variants at the *DQA1*01* gene, when not distinguishing between these two DQ types they are often referred to as DQw1. Similarly when not distinguishing between DQ7 and DQ8, the term DQw3 is used.

Table 1.3. Common HLA-DQ types referred to in this study. Figures in red refer to approximate gene frequency in a Caucasian population. Genotypes that are underlined are the most common of several listed.

Serotype		***Genotype***	
		DQB1*	***DQA1****
DQw1 (0.4)	DQ5	*0501, 0502 etc*	*0101* (0.1), *0102*
	DQ6	*0601, 0602 etc*	*0102* (0.2), *0101*
DQ2 (0.2)	DQ2	*0201, 0202*	*0201*
DQw3 (0.3)	DQ7	*0301*	*0501* (0.12)
	DQ8	*0302*	*0301* (0.14)
DQ4 (0.1)	DQ4	*04*	

1.2.3 Superantigen interactions with MHC class II

Superantigens bind to the class II molecule in several different ways but do so by utilising either or both of only two sites. On the class II alpha chain, a site on the distal α1 domain interacts with the N-terminal domain of superantigens. On the class II beta chain, a zinc dependent interaction, based around a highly conserved histidine residue at position 81, occurs with sites in the superantigen C-terminal domain, on the opposite side of the molecule from the low-affinity N-terminal domain site (Fig. 1.2). Superantigens have evolved several different modes of interaction with the class II molecule based on these two binding sites. One group of superantigens exemplified by SEB but including SSA and SPEA, utilise only the class II α-chain binding site. A second group, exemplified by SEA and including SED and SEE, utilise both the α- and β-chain binding sites and, in doing so, cross link class II molecules at the cell surface. SED may under some circumstances vary this mode of binding by forming homodimers through its C-terminal domain and binding class II only through the α-chain site, thus cross linking class II (Sundstrom, Abrahmsen *et al.* 1996). A third group, typified by SPEC and SMEZ, utilise only the β-chain binding site. Additionally these superantigens dimerise via their N-terminal domains and thus also achieve cross linking of class II at the cell surface. TSST-1 appears to adopt a fourth, unique interaction with the class II molecule through the α-chain but extending over the peptide binding groove and making contact with antigenic peptide. The sites of superantigen binding to MHC class II are illustrated in figure 1.3 (Fraser, Arcus *et al.* 2000). The families of bacterial superantigens grouped by mode of class II binding are set out in table 1.4.

Table 1.4. Classification of bacterial superantigens by MHC class II binding mode

Toxin	MHC class II α chain binding site	MHC class H β chain binding site
SEB SSA SEC1-3 SPEA	MHC α-chain binding site in N-terminal domain Low affinity No contact with peptide	Not present
TSST-1	MHC α-chain binding site in N-terminal domain Interacts with peptide Contacts two residues on β-chain	Not present
SEA SED SEE	MHC α-chain binding site in N-terminal domain	High affinity Zn dependent MHC β-chain binding site in the C-terminal domain Cross link class II
SPEC SMEZ SMEZ-2 SPEH SEH	MHC α-chain binding site obscured and non-functional	2nd high affinity Zn dependent site in N-terminal domain (His35, Glu54) allows dimerisation which obscures low affinity site Cross link class II

Although superantigen binding sites on the MHC class II molecule are located away from the most polymorphic regions of the molecule which are involved in binding of peptide antigen (Dellabona, Peccoud *et al.* 1990), MHC class II differences in both binding and presentation of superantigens are well established. The bacterial superantigens are highly species specific for man. Differences between murine and human responses to bacterial superantigens relate to differences in class II. Binding

affinities of bacterial superantigens for HLA class II are higher than for mouse MHC class II. Both *in vitro* and *in vivo* responses to bacterial superantigens are muted in mice but studies using mice transgenic for human class II have demonstrated a marked increase in superantigen responses as a result (Yeung, Penninger *et al.* 1996; Sriskandan, Unnikrishnan *et al.* 2001). In addition to such interspecies differences, differences between HLA class II isotypes in binding and presentation of superantigens exist. Most of the staphylococcal superantigens have higher affinities for HLA-DR than –DQ and elicit more marked T cell responses when presented by HLA-DR. Conversely some of the streptococcal superantigens preferentially utilise HLA-DQ (Norrby-Teglund, Nepom *et al.* 2002). Utilization of HLA-DP by superantigens seems to be universally poor.

It may be that the definition of superantigens as not being subject to HLA class II restriction has fostered the assumption that allelic differences in class II can not impact on superantigen presentation. Since the late 1980s-early 1990s when several studies failed to identify major allelic differences in class II binding of bacterial superantigens (Herman, Croteau *et al.* 1990; Mollick, Chintagumpala *et al.* 1991) the possibility has not been studied. More recently however, the crystal structures of several superantigens co-crystallised with MHC class II have been published and indicate that superantigen binding sites on the class II molecule, both on the α and β-chains, lie at sites which either are polymorphic or are likely to be conformationally altered by sub-isotype HLA class II polymorphisms (Jardetzky, Brown *et al.* 1994; Kim, Urban *et al.* 1994; Sundberg and Jardetzky 1999).

1.3 The T cell Receptor

1.3.1 General considerations

The T cell receptor (TCR) is a heterodimeric glycoprotein consisting of α and β chains linked by a disulphide bond. α-chain (*TRA)* genes include Variable (V), Joining (J) and Constant (C) gene segements and there are multiple loci of the V and J genes. β-chain (*TRB*) genes include the same V, J, and C genes with an additional Diversity (D) gene segment (Davis 1990). T cell receptor diversity is generated in part by somatic rearrangement of V, J, and C segments of *TRA* and V, D, J and C segments of *TRB*. The overall structure of the TCR (Fig. 1.3) excepting the Cα domain, resembles an antibody Fab fragment such that each of the α and β chains consists of canonical , immunoglobulin-like variable and constant domains, with three hypervariable complementarity determining regions (CDRs) from the two variable domains (Vα and Vβ) forming the ligand binding site for the peptide-MHC complex. Less variable framework regions (FRs) are interspersed. CDR1 and CDR2 are encoded solely within the V genes while CDR3 is encoded by V, D and J elements. Further diversity of CDR3 is generated by flexibility of VDJ recombination and by nucleotide insertions but somatic mutation is not a feature of TCR diversity. CDR3 is thus the most variable and contributes most to the peptide specificity of the TCR (Hennecke and Wiley 2001). There are 62-65 Variable segments in the β-locus (*TRBV*) which can be grouped into 30 subfamilies of one or more members. 39-41 *TRBV* genes are functional. Subfamilies are defined by sharing >75% nucleotide sequence homology (Folch and Lefranc 2000). Several different nomenclatures for classifying TCR Vβ have been proposed (Wei, Charmley *et al.* 1994; Arden, Clark *et*

al. 1995; Rowen, Koop *et al.* 1996). The International Immunogenetics Database (IMGT) classification of *TRBV* genes is now the standard genetic nomenclature (Folch and Lefranc 2000). The most commonly used serological system in use is that proposed by Arden (Arden, Clark *et al.* 1995). This is used by manufacturers of Vβ antibodies and will be used here. Appendix 1 shows the amino acid sequences for expressed human TCR Vβs by the Arden and IMGT systems.

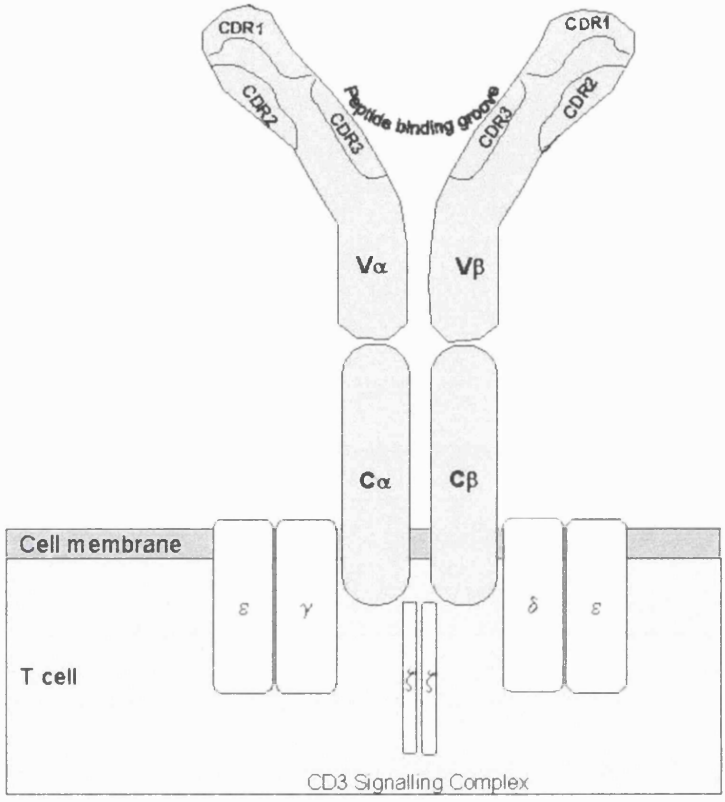


Fig. 1.3. General structure of the T cell receptor. The overall structure of an α,β T cell receptor is shown in association with the transmembrane CD3 signalling complex. The constant (C) and variable (V) regions of each chain are shown as are the locations of the Complementarity Determining Regions (CDR) 1-3.

1.3.2 Superantigen interactions with the T cell receptor

Superantigen binding to the T cell receptor is through the variable region of the β-chain (Herman, Kappler *et al.* 1991; Janeway 1991). Other parts of the TCR do not contribute directly to the interaction. However, in the altered orientation of TCR and MHC class II which is thought to result from superantigen binding, interactions between Vα and MHC class II are thought to take place and to confer some Vα specificity on the interaction (Reinherz, Tan *et al.* 1999). One recent report has challenged the tenet that bacterial superantigen interactions with TCR must be through the Vβ region. Petterson *et al* demonstrated purely Vα specific changes in T cell repertoire following stimulation by SEH (Petersson, Pettersson *et al.* 2003).

Several crystal structures of superantigens in complex with TCR have now been published (Malchiodi, Eisenstein *et al.* 1995; Li, Llera *et al.* 1998; Sundberg, Li *et al.* 2002). The areas of the superantigen structure in contact with TCR Vβ lie between the two main domains of the molecule and incorporate the α2 helix, β2-3 loop, β4 strand, β4-5 loop, β5 strand and α5 helix (Fig. 1.2) (Papageorgiou and Acharya 1997). Sites on the TCR Vβ region in contact with superantigen are concentrated in CDR1 CDR2 and FR3 (Pullen, Wade *et al.* 1990; Pullen, Bill *et al.* 1991), although the relative contribution made by each of these regions to the overall interaction varies for the superantigens studied thus far. Figure 1.4 shows the regions of the TCR variable region which make contact with peptide and demonstrates how superantigen binding to the TCR is located lateral to the peptide binding groove. Additionally some superantigens such as SEC and SEB appear to make most of their contacts with TCR main chain atoms while SPEA makes significant contact with side chain atoms.

The Vβ specificity of the interaction with the TCR for SEB and SEC is likely therefore to result from conformational factors while sequence differences might make a significant impact on SPEA interactions (Bentley and Mariuzza 1996). Since a superantigen will generally bind several different Vβ regions and trigger responses in all T cells of those Vβ families, 25-50% of resting T cells may be activated in this way (Choi, Herman *et al.* 1990). Each superantigen is associated with its characteristic pattern of Vβ specific T cell activation sometimes referred to as the Vβ signature (Kappler, Kotzin *et al.* 1989).

1.4 The microbial superantigens

1.4.1 Bacterial superantigens

Over the last five years the list of known bacterial superantigens has grown considerably, mainly as a result of the sequencing of several bacterial genomes (Ferretti, McShan *et al.* 2001; Beres, Sylva *et al.* 2002; Smoot, Barbian *et al.* 2002). There are now 41 established bacterial superantigens described in the literature. In addition to the superantigens of *S. pyogenes* and *S. aureus* (table 1.5) superantigens have now been identified in *Streptococcus equi*, and *Streptococcus dysgalactiae* (Miyoshi-Akiyama, Zhao *et al.* 2003; Proft, Webb *et al.* 2003). It is interesting to note that the two organisms which exhibit expression of superantigens most extensively by far; *S. aureus* and *S. pyogenes*, are, among gram positive bacteria, the most virulent and species specific pathogens of man. Analysis of the shared structure and sequence features of these toxins demonstrates how they have evolved within several sub families (Fig. 1.5). Five families or groups are defined in this family tree, with TSST-1 forming a separate branch of its own. The recently described *Streptococcus equi* superantigens are also included (Proft, Webb *et al.* 2003). The correspondence between these families and the classification of superantigens by their MHC class II binding mechanism (table 1.3) is striking and suggests a role for interactions with the class II molecule having driven superantigen evolution.

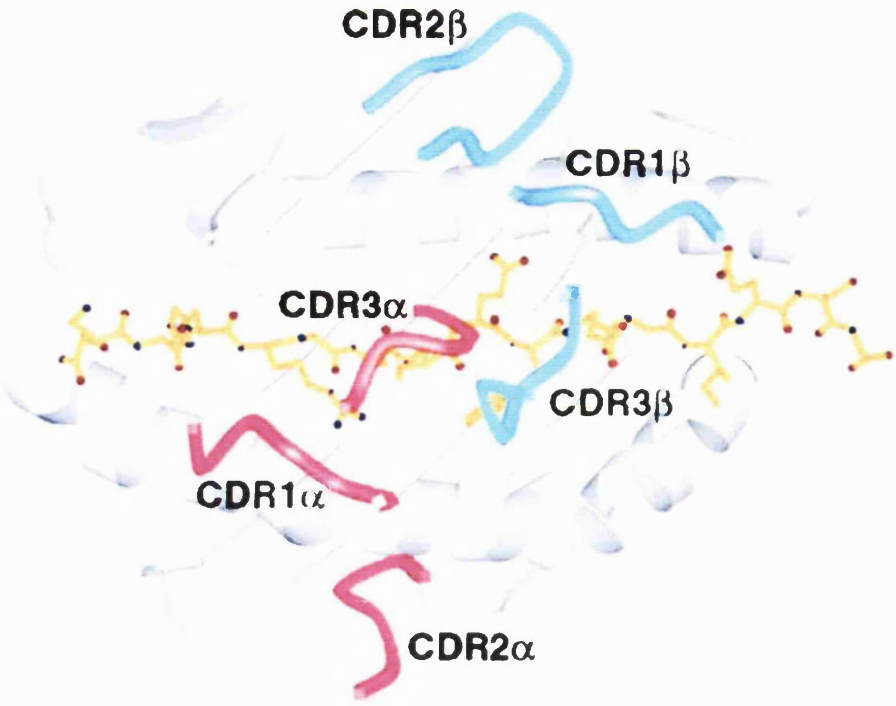


Fig. 1.4. TCR orientation over the peptide MHC surface. The interaction of the VDJ encoded CDR3 regions with peptide is demonstrated. Peptide lying in the MHC class II binding groove is shown in yellow, TCR α-chain in pink and β-chain in blue. The location of CDR2β, the main site superantigen binding to the TCR, is demonstrated lying lateral to the peptide/MHC complex. Adapted from Hennecke, Wiley 2001 after Reinherz *et al* 1999.

Most bacterial superantigens are coded for on phages. In *S. pyogenes* the genes for only SPEG and SMEZ appear to be chromosomally located (Proft, Moffatt *et al.* 2000). Phages also code for a range of other secreted proteins such as DNases and the phospholipase Sla. Several phages are found in most strains and are responsible for most of the genetic variation between strains (Banks, Beres *et al.* 2002).

A number of streptococcal and staphylococcal secreted proteins have mistakenly been regarded as superantigens because when analysed in purified form they were contaminated by traces of highly potent superantigens. SPEB, now known to be a cystein protease, SPEF, now known to be identical to DNAse B, streptococcal M protein and exfoliative toxins A and B of *S. aureus* are all examples of this (Plano, Gutman *et al.* 2000; Sriskandan, Unnikrishnan *et al.* 2000; Gerlach, Schmidt *et al.* 2001).

Although the most well studied bacterial superantigens are those of the gram positive cocci, other pathogens also produce proteins with superantigen properties. *Mycoplasma arthritidis*, an organism responsible for producing arthritis in rodents, produces a 21.3kD protein, *M. arthritidis* Mitogen (MAM) which triggers Vβ specific T cell activation although it is only distantly related to the other bacterial superantigens (Cole, Knudtson *et al.* 1996). The interaction of MAM with the T cell receptor appears to be unusual in that MAM interacts with CDR3 and with amino acids determined by Jβ and Dβ genes (Hodtsev, Choi *et al.* 1998). *Yersinia pseudotuberculosis* is a gram negative bacillus, infection with which is associated with gastrointestinal symptoms but also symptoms resembling superantigen toxicity including generalised erythema, conjunctivitis and desquamation. *Y.*

pseudotuberculosis mitogen (YPM) is about half the molecular weight of, and has no sequence homology with, other superantigens but nevertheless stimulates polyclonal, HLA class II dependent and Vβ specific, T cell proliferation (Abe and Takeda 1997; Ito, Seprenyi *et al.* 1999). A novel bacterial superantigen I2, of unknown bacterial origin which stimulates mVβ5 independent of antigen processing and appears to be structurally unrelated to either MMTV superantigens or the pyrogenic exotoxin superantigens has recently been identified as a trigger for Crohn's disease (Dalwadi, Wei *et al.* 2001).

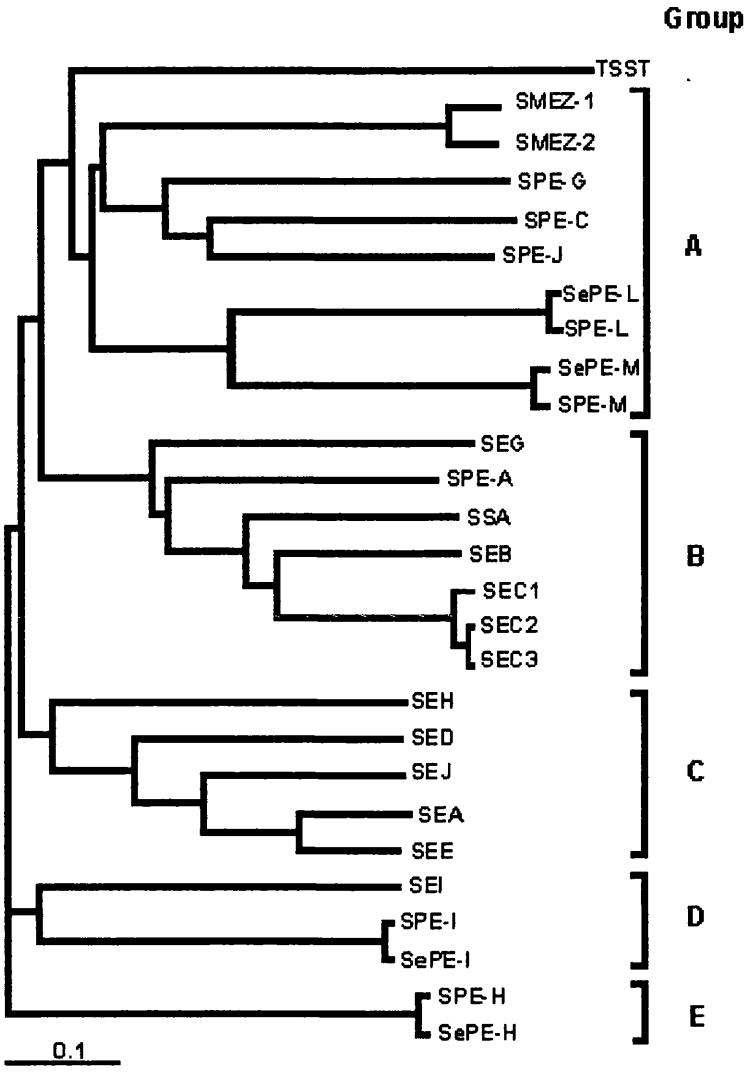


Fig. 1.5. Family tree of the bacterial superantigens. Five structurally related sub-families arbitrarily labelled A-E are discernable, with only TSST-1 not falling within one of these. The recently described *S. equi* Pyrogenic Exotoxin superantigens are also included (SePE). The tree was derived from multiple sequence alignments. (Proft, Webb *et al* 2003).

Table 1.5. Important Features of the Superantigens of *S. aureus* and *S. pyogenes*.

SAg	Mwt	Gene location	Class II binding	MHC affinity	TCR affinity	P50 (pg/ml)	Human TCR Vβ specificity
SEA	27.1	Phage	DR α, β, peptide	13nM		0.1	1, 5, 8, 9, 16, 22
SEB	28.4	Phage	DR α	14μM	140μM	0.8	3, 12, 14, 17
SEC1	27.5	Phage	DR α		0.9μM	0.2	3, 6, 12, 15
SEC2	27.6	Phage	DR α			0.2	12, 13, 14, 17, 20
SEC3	27.6	Phage	DR α	48μM	4.5μM	0.2	5, 12
SED	26.3	Phage	DR α, β				1, 5, 12
SEE	26.4	Phage	DR α, β			0.2	5, 6, 8
SEG	27.0	Chromo	?				3, 12, 14, 15
SEH	25.2	Chromo	DR α, β, peptide				?
SEI	24.9	Chromo	?				1, 5, 23
SEJ-Q	28.5	Phage	?				?
TSST-1	22.0	Phage	DR α, peptide			0.2	2
SPEA	25.8	Phage	DQ α	104nM	6.2μM	10	12, 14
SPEC	24.4	Phage	ββ			0.1	2, 3, 15
SPEG	24.6	Chromo	β			2	2, 4, 9
SPEH	23.6	Phage	β			50	2, 7, 9, 23
SPEI-M	-	Phage	β ?α			1-10	
SSA	26.9	Phage	α				1, 3, 15
SMEZ	24.3	Chromo	β			0.08	2, 4, 8
SMEZ-2	24.2	Chromo	β			0.02	4, 8

Data are broadly derived from (Fraser, Arcus *et al.* 2000) and (Proft and Fraser 2003), with additional data from (Hudson, Tiedemann *et al.* 1995; Li, Llera *et al.* 1998). P50 = concentration producing half maximal proliferation of human PBMCs.

1.4.2 The cellular response to superantigens

The functional consequences of T cell activation by superantigens are complex. Initial T cell activation is associated with Vβ specific proliferation and production of lymphokines and monokines, including IL-1, IL-2, IL-6, TNF-α and IL-10 (Fast, Schlievert *et al.* 1989; Hackett and Stevens 1993; Kotzin, Leung *et al.* 1993). The role of T cells in this process was proven in experiments in which mice lacking T cell function, either genetically or because of cyclosporin treatment, were shown to be resistant to superantigen toxicity (Marrack, Blackman *et al.* 1990). A protective effect from blockade of both TNFα and IFN-γ has been demonstrated *in vivo* (Miethke, Wahl *et al.* 1992; Florquin, Amraoui *et al.* 1994; Stevens, Bryant *et al.* 1996). While proliferation of targeted TCR Vβs is observed *in vitro*, this expansion may be transient or absent *in vivo*, and is followed by a marked reduction in numbers of targeted TCR Vβs. Following *in vivo* stimulation by a superantigen, T cells of the targeted Vβ types are anergic to further stimulation whether by superantigen, peptide antigen or antiCD3 (MacDonald, Baschieri *et al.* 1991; Baschieri, Lees *et al.* 1993; Sriskandan, Unnikrishnan *et al.* 2001). Under some circumstances complete deletion of targeted Vβs may occur following *in vivo* T cell stimulation. For example, superantigen stimulation of immature T cells in the developing thymus, of in bred mice strains which express endogenous MMTV superantigens, may result in complete deletion by apoptosis of targeted Vβ TCRs from the T cell repertoire (Kappler, Roehm *et al.* 1987; Kappler, Staerz *et al.* 1988; MacDonald, Schneider *et al.* 1988). Deletion of superantigen responsive Vβs has also been detected *in vivo* following exposure to bacterial superantigens (Wahl, Miethke *et al.* 1993).

In addition to activating T cells, superantigens also activate the APCs involved in superantigen presentation. Following superantigen binding to MHC class II at the cell surface, intracellular signal transduction pathways are activated and as a result IL-1 and TNF-α gene transcription occurs (al-Daccak, Mehindate *et al.* 1994).

1.4.3 Other properties of bacterial superantigens

Augmentation of endotoxin activity

The toxic effects of bacterial superantigen and endotoxin are dramatically synergistic. Their co-administration to experimental animals is associated with a reduction of the LD_{50} of either toxin given alone by up to 50,000 fold (Schlievert 1982). Studies using mutated forms of superantigens suggest this property is distinct from superantigenicity. The implications of endotoxin-superantigen synergy for the clinical impact of superantigen production are two fold. First, the circulation of bacterial superantigen may reduce the threshold for endotoxicity to a degree at which circulation of endogenous endotoxin may be sufficient to contribute to shock. Secondly, coexistence of gram positive and gram negative organisms, for instance as 'colonisation' of critically ill patients may through a 'double hit' of endotoxin and superantigen result in shock in the absence of a typical focus of bacterial infection (Bannan, Visvanathan *et al.* 1999).

Emesis

The staphylococcal exotoxins SEA-E and SEG-I, as well as being superantigens, are potent gastrointestinal toxins responsible for staphylococcal food poisoning. The precise mechanism by which the SEs induce vomiting has not been established. Although the superantigenicity and gastrointestinal toxicity properties of the SEs are

located at distinct sites on the molecule there is a close correlation between emetic and superantigen activities (Alber, Hammer *et al.* 1990). The majority of structural mutations disrupt both properties (Harris, Grossman *et al.* 1993). Nevertheless, not all bacterial superantigens are exotoxins. The explanation for this relates partly relates to physical properties. SEs are able to withstand degradation by the heat of cooking and pasteurisation, temperatures of above 120°C for 5-10 minutes are required to cause inactivation (Balaban and Rasooly 2000), and are resistant to degradation by pepsin. TSST-1 for example is sensitive to pepsin degradation and is not an enterotoxin. Other structural properties must also play a part since SPEA for example is heat and pepsin resistant but does not induce emesis on ingestion. Schlievert *et al* have recently proposed that the precise orientation of a loop in the superantigen N terminal domain may be the core structural feature required for emesis (Schlievert, Jablonski *et al.* 2000).

Local inflammation

A third action of bacterial superantigens is their ability to stimulate neutrophil recruitment to a site of infection. Again this effect appears to be independent of their superantigenicity since subcutaneous injection of staphylococcal enterotoxin B (SEB) in mice leads to neutrophil influx independent of T cell stimulation. The mechanism appears to be release of TNFα from monocytic cells which triggers upregulation of chemokines and adhesion molecules such as ICAM (Diener, Tessier *et al.* 1998). The importance of this process in infection remains to be determined but in mice, which by virtue of MHC class II differences are resistant to the superantigen effects of SPEA, infection by SPEA producing strains of *S. pyogenes* results in a more vigorous

neutrophil response and better outcome than infection by strains lacking the SPEA gene (Sriskandan, Unnikrishnan *et al.* 1999).

1.4.4 Mouse Mammary Tumour Virus superantigens and murine endogenous retroviral superantigens

The first superantigens to be identified, were not microbial toxins but were the minor lymphocyte stimulating (Mls) antigens expressed in thymic stromal cells of mice (Festenstein 1973). Mls antigens are now known to be encoded by genes integrated into the mouse genome from the genomes of the mouse mammary tumour viruses (MMTVs). MMTVs are retroviruses which exist either as infectious virus transmitted from mother to offspring during suckling (exogenous MMTV) or as a germ-line integrated provirus transmitted by genetic inheritance (endogenous MMTV). Exogenous MMTVs utilise a superantigen in a crucial stage of their viral life cycle. When the virus infects B cells the MMTV superantigen is expressed in association with MHC class II at the cell surface where superantigen reactive T cells are activated, providing 'help' for infected B cells. This leads in turn to a large increase in the number of infected B cells. This superantigen mediated increase in viral load is essential for establishing viral latency and transport of the virus to mammary tissue for transmission to the next generation. In in bred mouse strains where MMTVs have become integrated into the genome, MMTV superantigen expressed in the immature thymus causes complete deletion of TCR Vβ targets, so that the mice are born with 'holes' in the TCR Vβ repertoire. By virtue of this process these mice are completely resistant to infection by the strains of exogenous MMTV which target these Vβs. The efficacy of *mtv* superantigens in causing T cell deletion is MHC class II dependent.

H-2E molecules are superior to H-2A in presentation of all *mtv* superantigens. Some *mtv*s are H-2E dependent so that for example C57BL6 mice which express only H-2A, carry the endogenous retrovirus superantigens mtv8 and mtv9, yet do not delete Vβ11 and 12 (Luther and Acha-Orbea 1997). HLA class II transgenes can substitute for H-2E in presentation of H-2E dependent *mtvs* so that HLA class II transgenic mice strains may have *mtv* associated Vβ deletions which are not found in the parent strain (Altmann, Takacs *et al.* 1993).

1.4.5 Viral superantigens in human disease

Human equivalents of MMTVs have not been identified and the human T cell repertoire contains no Vβ deletions like those found in mice strains carrying *mtv*s. It has been known for some time that infection by two human herpes viruses, Epstein Barr Virus and Cytomegalovirus is accompanied by polyclonal Vβ specific T cell expansion indicating that a superantigen is present (Dobrescu, Ursea *et al.* 1995; Sutkowski, Palkama *et al.* 1996). In neither of these viruses have genes encoding for putative superantigens been identified. Recently this dilemma was resolved for EBV infection when it was demonstrated that the superantigen associated with EBV infection is encoded not by EBV but by a human endogenous retroviral gene *HERV-K18*. EBV infection induces expression of this gene and it may be that in a manner analagous to MMTV, EBV uses superantigen activated T cells to establish viral latency in infected B cells (Sutkowski, Conrad *et al.* 2001; Woodland 2002).

Superantigenicity has been demonstrated for the products of another human endogenous retrovirus family; HERV-W. HERV-W expression can be induced by *Herpes simplex* type 1 (HSV-1) infection. Both the expressed and a recombinant

protein stimulate Vβ16 and 17 proliferation (Perron, Jouvin-Marche *et al.* 2001). The observation that HERV-W is expressed in cell lines derived from patients with multiple sclerosis and not healthy controls has lead to the suggestion that HERV-W could explain the epidemiological link between viral infections including HSV and MS (Lafon, Jouvin-Marche *et al.* 2002). In addition to a direct effect of viral infections on expression of HERVs the inflammatory response itself may be able to induce expression of these superantigens, as IFN-α has been shown to regulate gene expression (Stauffer, Marguerat *et al.* 2001).

Among viruses causing human disease, an exogenous viral superantigen been identified only in rabies (Lafon, Lafage *et al.* 1992; Lafon, Scott-Algara *et al.* 1994). The rabies virus nucleocapsid protein has been shown to induce Vβ specific polyclonal T cell activation which is MHC class II dependent, but not MHC class II restricted. Atypically though, the T cell response to this superantigen is Th2 skewed (Martinez-Arends, Astoul *et al.* 1995).

1.5 Microbial superantigens and human disease

1.5.1 Toxic Shock Syndrome

In 1978 a paediatrician, James Todd, gave the first formal description of Toxic Shock Syndrome. He reported seven children with a toxin mediated, scarlet fever like illness accompanied by shock, renal failure and disseminated intravascular coagulation associated with superficial *S. aureus* infection (Todd, Fishaut *et al.* 1978). The disease first came to world-wide public attention in the 1980s when numerous cases of Toxic Shock were reported related to the use of high absorbency tampons and to a lesser extent with certain nasal surgical packs (Hajjeh, Reingold *et al.* 1999). In 1987,

at a time when, as a result of public health measures, the incidence of Staphylococcal Toxic Shock was waning, Lawrence Cone first described Streptococcal Toxic Shock syndrome. He reported two cases of a systemic illness indistinguishable from Staphylococcal Toxic Shock associated with invasive *S. pyogenes* infection (Cone, Woodard *et al.* 1987). It is likely that descriptions of toxic forms of severe streptococcal infection from the pre-antibiotic era refer to the same syndrome and that the world-wide spread of severe invasive streptococcal infections accompanied by toxic shock which took place during the 1990s was resurgence rather than the spread of a truly novel disease (Stevens 1994). Changes in the epidemiology of *S. pyogenes* strains isolated from invasive infection were identified during this period of time. In particular M1 and M3 strains became more prevalent (Holm, Norrby *et al.* 1992; Eriksson, Andersson *et al.* 1999) although a wide range of M types have been associated with severe invasive disease (Efstratiou 2000). Also a correlation was noted between severe invasive disease and strains carrying the phage mediated superantigens SPEA and SPEC (Musser, Hauser *et al.* 1991; Holm, Norrby *et al.* 1992; Demers, Simor *et al.* 1993; Talkington, Schwartz *et al.* 1993). In particular clones carrying the novel variants *speA2* and *speA3* became more common, while *speA1* was shown to be the predominant allele in clones from the early part of the twentieth century (Nelson, Schlievert *et al.* 1991; Musser, Kapur *et al.* 1993). Although SPEA-2 and SPEA-3 only differ from SPEA-1 by a single amino acid, they are more potent superantigens (Kline and Collins 1996).

While Staphylococcal and Streptococcal Toxic Shock syndromes share many clinical features set out in table 1.6, some distinguishing characteristics are of importance

both clinically and in understanding disease pathogenesis (Stevens 1996). Both syndromes are characterised by a flu-like prodrome which progresses over hours or days to development of fever with generalised erythema and frequently conjunctival injection. Prior to development of shock, patients often do not appear to be severely ill but investigations will show a very marked inflammatory response, often deranged liver and renal function and hypoalbuminaemia. Fulminant cardiovascular shock then develops accompanied by multi-organ failure, disseminated intravascular coagulation, hypoalbuminaemia and generalised and pulmonary oedema. In those who survive. desquamation of the palms and soles occurs approximately two weeks after the initial infection (McCormick, Yarwood *et al.* 2001). One important difference between Staphylococcal and Streptococcal toxic shock is the sites of underlying infection. Staphylococcal toxic shock almost invariably follows on from superficial infection, either of the skin, at a surgical site or, most infamously, from vaginal colonisation during menstruation. Menstrual toxic shock appears only to occur in the context of colonisation by TSST-1 producing strains of *S. aureus* because of the distinctive ability of TSST-1 to cross intact mucus membranes (Schlievert, Jablonski *et al.* 2000). Streptococcal toxic shock in contrast, very rarely follows superficial infection of the pharynx or skin but is usually a sequel of invasive infections such as necrotising fasciitis (Cockerill, MacDonald *et al.* 1997). In keeping with this difference in the site of infection, staphylococcal toxic shock is rarely accompanied by bacteraemia while in streptococcal TSS bacteraemia is common. The mortality of the two forms of toxic shock, which is about 5% for Staphylococcal TSS and 50% for Streptococcal TSS, reflects this difference in the severity of the underlying disease.

Table 1.6. Case definitions of Streptococcal and Staphylococcal Toxic Shock Syndromes

Streptococcal Toxic Shock (CCDC 1996)	**Staphylococcal Toxic Shock (CCDC 1997)**
Isolation of *S. pyogenes* ('definite' STSS if from a sterile site)	Fever ≥ 38.9
Hypotension <90mmHg	*Hypotension* <90mmHg
Multisystem involvement ≥ 2 of... *Renal impairment*: raised creatinine *Coagulopathy*: Platelets < 100,000/mm^3 or disseminated intravascular coagulation,. *Liver involvement*: raised transaminases or bilirubin. *Acute respiratory distress syndrome* *Generalised erythema* +/- desquamation *Soft tissue necrosis* including necrotising fasciitis or myositis	Multisystem involvement with ≥ 3 of... *Gastrointestinal*: vomiting or diarrhoea at onset of illness *Muscular*: severe myalgia or raised creatine phosphokinase *Mucous membrane*: vaginal, oropharyngeal, or conjunctival hyperemia *Renal*: raised creatinine or pyuria without infection *Hepatic*: raised transaminases or bilirubin *Hematologic*: platelets < 100,000/mm^3 *Central nervous system*: disorientation or altered consciousness *Generalised erythema* +/- desquamation

Notwithstanding the evidence that microbial factors play a central role in the causation of STSS, it is also well recognised that toxigenic clones of *S. pyogenes* can be isolated from the whole spectrum of syndromes associated with *S. pyogenes* infection from asymptomatic carriage, through trivial superficial infections, to life threatening invasive disease and STSS (Chatellier, Ihendyane *et al.* 2000; Johnson, Wotton *et al.* 2002). Were toxic shock syndrome primarily the result of infection by novel virulent clones of bacteria, the epidemiology of the disease would be characterised by outbreaks and a high secondary attack rate. In fact the epidemiology of severe streptococcal infection, with a few notable exceptions such as the well-described Gloucestershire outbreak of 1995 (Cartwright, Logan *et al.* 1995), is characterised by isolated cases (Basma, Norrby-Teglund *et al.* 1999; Robinson, Rothrock *et al.* 2003). The close household contacts of cases of TSS are frequently found to be colonised with the organism which caused disease in the case but are rarely effected (Robinson, Rothrock *et al.* 2003). Host predisposition to the toxic effects of superantigens must therefore play a large part in determining the outcome of infection by toxigenic strains of *S. pyogenes*. The presence or absence of humoral immunity to superantigens, developed for example during a previous superficial infection, might explain host predisposition to toxic shock during invasive streptococcal infections. Recurrence of menstrual toxic shock is associated with failure to develop neutralising antibody to TSST-1 (Stolz, Davis *et al.* 1985). However other forms of both Staphylcoccal and Streptococcal toxic shock are rare (Andrews, Parent *et al.* 2001). When Basma *et al* looked for such an effect in *S. pyogenes* infection, antibodies appeared to be protective against invasive disease but

not against toxic shock in patients with invasive disease (Basma, Norrby-Teglund *et al.* 1999).

A causative role for superantigens in toxic shock syndrome has been most conclusively demonstrated for TSST-1 in Staphylococcal, menstrual toxic shock. Comparing features of uterine infection in a rabbit model of Staphylococcal Toxic Shock using isogenic strains of bacteria, either toxin negative or carrying the *tst* gene, De Azavedo *et al* demonstrated the importance of TSST-1 in production of toxin mediated features of toxic shock (de Azavedo, Foster *et al.* 1985). Streptococcal superantigens have been identified in the serum of patients with toxic shock but not invasive disease without shock (Sriskandan, Moyes *et al.* 1996; Proft, Sriskandan *et al.* 2003). However, the implied role of other superantigens in the pathogenesis of Staphylococcal and Streptococcal Toxic shock is still largely inferred from the data concerning TSST-1, with the exception that a role for SPEA was demonstrated in an HLA-DQ transgenic mouse model of Streptococcal TSS using isogenic *spea*+ and *spea*- strains of *S. pyogenes*. (Sriskandan, Unnikrishnan *et al.* 2001).

1.5.2 Food Poisoning

Staphylococcal enterotoxins rank as the second most common cause of food poisoning in the developed world (Altekruse, Cohen *et al.* 1997). The syndrome of Staphylococcal food poisoning is distinctive. A short incubation period of 2-6 hours is followed by nausea and vomiting, which may be profuse. The diarrhoeal component of the syndrome occurs later, is generally mild and the whole illness last around 24 hours. Death is rare and occurs only in the debilitated at the extremes of age. SEA is the most commonly implicated toxin followed by SED and SEB. Only

tiny quantities of toxin (<1μg) are required to cause symptoms and only 10^3 organisms per gram of food are required to produce detectable levels of toxin (Balaban and Rasooly 2000).

1.5.3 Kawasaki's Disease

Kawasaki's disease (or mucocutaneous lymph node syndrome) is a multisystem vasculitis most common in children aged 6 months to 8 years which occurs in spring epidemics. Many clinical features of Kawasaki's disease are reminiscent of toxic shock: erythema, conjunctivitis, a very marked inflammatory response and subsequent desquamation. Acute Vβ-specific changes (particularly Vβ2 expansion) have been observed in many cases (Abe, Kotzin *et al.* 1993; Curtis, Zheng *et al.* 1995; Leung, Meissner *et al.* 1995). Autoantibodies to cardiac muscle and vascular endothelium have been demonstrated (Cunningham, Meissner *et al.* 1999; Suzuki, Muragaki *et al.* 2002). Following the acute illness, up to 25% of children develop aneurysmal dilatation of the coronary arteries. Although no single superantigen has been implicated and there is still considerable debate about the precise aetiology of Kawasaki's disease, the condition is probably the outcome of exposure to one of a number of superantigens in a susceptible host (Barron, Shulman *et al.* 1999). Recently, using a mouse model of Kawasaki's disease arteritis triggered by *Lactobacillus casei* infection, it was demonstrated that superantigenic activity correlated directly with induction of coronary arteritis (Duong, Silverman *et al.* 2003).

1.5.4 Psoriasis and atopic eczema

Stimulation of PBMCs with superantigens, in addition to the changes described above, leads to upregulation of cutaneous lymphocyte associated antigen (CLA) which is a receptor involved in migration of T cells to skin (Leung, Gately *et al.* 1995).

Psoriasis is a common inflammatory disease of the skin characterised by keratinocyte proliferation and eruption of red, scaled maculopapules. One major form of the disease, guttate psoriasis, is often preceded by streptococcal pharyngitis, specifically involving strains of *S. pyogenes* producing SPEC (Telfer, Chalmers *et al.* 1992). Expansion of Vβ2 T cells in the skin of psoriatic lesions further implicates a superantigen in the aetiology (Leung, Travers *et al.* 1995).

Atopic eczema is also a genetically determined, chronic skin disease characterised by inflammation. Dysfunction of macrophages and T cells are thought to play a role and overproduction of IL-4, IL-5 and IgE are characteristic features. Although there are scattered case reports in the literature of superantigen exposure apparently triggering atopy (Schlievert 1993; Michie and Davis 1996), the best evidence for a role for superantigens in eczema is in its exacerbation. Eczematous lesions are frequently colonised with toxigenic strains of *S. aureus* and penetration of toxins through inflamed skin may allow activation of vascular epithelium, upregulation of adhesion molecules and exacerbation of the inflammatory response by activated T cells expressing CLA (Bunikowski, Mielke *et al.* 2000).

1.5.5 Superantigens and induction of autoimmunity

The ability of superantigens to expand large numbers of T cells without regard to TCR antigen specificity raises the possibility that subpopulations of expanded cells could be specific for self-antigen (Kotzin, Leung *et al.* 1993). Such a mechanism has been proposed as a trigger for MS (Rudge 1991). Treatment of PBMCs from MS patients and healthy subjects with staphylococcal superantigens has been shown to expand T cells with specificity for a range of myelin epitopes (Zhang, Vandevyver *et al.* 1995). Direct evidence for superantigens triggering autoimmunity however is scarce. In the murine model of Multiple Sclerosis, Experimental Allergic Encephalomyelitis (EAE), disease can be induced in susceptible animals by exposure to an N-terminal peptide of myelin basic protein (MBP). The majority of T cells recognising this peptide are Vβ8. Administration of SEB, which targets murine Vβ8, to susceptible mice which have been immunised with the MBP peptide, causes relapses and exacerbation of EAE, although SEB does not initiate EAE in unimmunised mice (Brocke, Gaur *et al.* 1993). Injection of MAM into mice recovering from collagen-induced arthritis can induce disease relapse or initiate disease in mice where immunisation had initially induced no disease (Cole and Griffiths 1993). So while there is some evidence that superantigens may exacerbate an established autoimmune process, evidence that superantigens may induce autoimmunity *de novo* is lacking.

Although autoimmune disease has not been reported as a sequel of toxic shock syndrome, superficial *S. pyogenes* infection is a well established trigger of autoimmunity. Rheumatic fever is a syndrome of carditis, migratory polyarthritis and

chorea which follows about 3-6 weeks after and episode of streptococcal pharyngitis. Autoreactive antibodies and T cells detected in rheumatic fever cross-react with streptococcal components such as M protein and this is widely accepted as the mechanism underlying the development of the disease (Cunningham 2000). Poststreptococcal Glomerulonephritis usually occurs 2-3 weeks after streptococcal pyoderma and is generally ascribed to either immune targeting of streptococcal antigens 'planted' in the renal basement membrane, or molecular mimicry (Oliveira 1997; Cunningham 2000). In both these syndromes, as in Kawasaki's disease, it is plausible that superantigen induced autoimmunity could play a role in aetiology. Three of the most recently identified streptococcal superantigens SPE-K, SPE-L and SPE-M have identified in different, M3 and M89, rheumatigenic clones of *S. pyogenes*. Circulating antibodies to these superantigens were detected in sera of effected patients (Smoot, McCormick *et al.* 2002; Proft, Webb *et al.* 2003). Interestingly these superantigens each target the same Vβ: Vβ1.

1.5.6 HLA associations with superantigen mediated disease

One recently published study has demonstrated that an HLA association with superantigen mediated shock exists (Kotb, Norrby-Teglund *et al.* 2002). In this study Kotb and co-workers were looking at invasive *S. pyogenes* infections in previously healthy people. They compared HLA class II haplotypes in invasive cases with and without features of severe streptococcal disease and a population matched cohort of healthy people. The principal finding of the study was an association of certain HLA haplotypes (*DRB1*14/DQB1*0503*) with predisposition to, and (*HLA-DRB1*1501/DQB1*0602*) with protection from, severe streptococcal disease.

Although this was fundamentally an epidemiological study it also demonstrated that HLA class II haplotypes associated with severe disease were associated with greater proliferation of PBMCs and purified T cells in response to partially purified culture supernatants in the presence of HLA homozygous B-LCLs. Being designed to search for an association between severity and HLA haplotype this study was unable to address the question of what the underlying mechanisms might be. The patients studied will necessarily have been infected with different *S. pyogenes* strains each carrying multiple different Superantigen genes (Ferretti, McShan *et al.* 2001). Similarly the culture supernatants used to stimulated responses in vitro will have contained multiple superantigens. Consequently no specific relationship between an individual superantigen and the class II involved in its presentation could be studied. The authors speculated that the association between class II haplotype and susceptibility may be through modification of the inflammatory response to superantigens.

A number of studies have looked for an HLA association with Kawasaki's Disease in different populations. The results are unclear and where associations have been found these seem to be linked to particular population groups (Matsuda, Hattori *et al.* 1977; Kato, Kimura *et al.* 1978; Krensky, Berenberg *et al.* 1981; Keren, Danon *et al.* 1982; Barron and Silverman 1992; Fildes, Burns *et al.* 1992). In view of the lack of correlation of Kawasaki's disease with any individual superantigen this disparity of results in epidemiological studies is probably not surprising. A similar situation exists for rheumatic fever where various associations have been reported including with HLA-DR4 and HLA-DR2 in North America, irrespective of racial background, with

DR7 in Brazil and *DQA1*0102* in china (Ayoub, Barrett *et al.* 1986; Olmez, Turgay *et al.* 1993; Gu, Yu *et al.* 1997; Visentainer, Pereira *et al.* 2000). Such observed associations might be due to class II genes lying close to, or in linkage disequilibrium with, a putative rheumatic fever susceptibility gene (Stollerman 2001). An alternative explanation is that rheumatic fever is the common end point of different infective triggers present in different populations.

1.6 Aims of this project

The work described in this thesis was originally stimulated by the question of why invasive infection by identical, toxigenic strains of *S. pyogenes* only causes toxic shock in a minority of patients. Could it be that in addition to any differences which may exist in the adaptive immune response, genetic differences in the response to superantigen toxins might exist? The work has focused on HLA class II as the site at which interactions with superantigens may differ because the fundamental interaction which superantigens make with the immune system is with the MHC class II molecule and the genes encoded by the HLA region are the most polymorphic in the human genome. Furthermore the lines of experimental evidence and theoretical considerations which have already been discussed suggest that MHC class II polymorphisms could determine superantigen responses.

The specific aims of the project were,

- to determine whether differences in binding of superantigens to HLA class II exist and
- to define the functional implications of any such differences on the T cell response.

Secondary aims which developed out of observations made during the course of the work were,

- to characterise the Vβ specific T cell response to superantigens in detail and
- to study the impact of bacterial superantigens on autoreactivity of T cells.

CHAPTER 2

Materials and General Methods

2.1 Materials

General material and methods applicable to work carried out through out this project are described here. Techniques developed during the course of this work are described in detail in the experimental chapters which follow.

2.1.1 Standard media and solutions

Luria Bertrani (LB) broth:	1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH7.5
Phosphate buffered saline (PBS)	10mM sodium phosphate, 0.9% sodium chloride
Running buffer (SDS PAGE)	25mM Tris, 192mM glycine, 0.1% SDS
1x SDS sample buffer	50mM Tris HCL pH6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol
Coomassie Blue Stain	0.25 Coomassie brilliant blue R250 in 40% methanol, 10% acetic acid
De-staining solution	40% methanol, 10% acetic acid
Ponceau S Stain	2% Ponceau S, 30% trichloroacetic acid, 30% sulfosalicylic acid
Transfer buffer	25mM Tris, 192mM glycine, 20% methanol
FACs buffer	PBS, 1% BSA, 0.01% sodium azide
Complete RPMI (tissue culture)	RPMI 1640, 10% fetal calf serum, 2mM L-glutamine, 50U/ml penicillin, 50μg/ml streptomycin

2.1.2 Toxins

Purified and recombinant SPEA, SEA, SEB and biotin conjugates of these toxins were purchased from Toxin Technology Inc (Sarasota FL).

Recombinant SPEA-1 was expressed using a pET expression system as described in chapter 3.

2.1.3 Antibodies

Table 2.1. HLA class II antibodies used in this study.

Antibody	Species	Source	Specificity	Application
L2	Mouse	Hybridoma supernatant	Pan DQ α-chain	FACS
Leu10	Mouse	Becton Dickenson, San Diego CA	DQ1, 3 β-chain	FACS
SPV-L3	Mouse	Hybridoma supernatant	Pan DQ α-chain	FACS/ELISA
L243	Mouse	Hybridoma supernatant	DR α-chain	FACS, T cell purification
Sc17963	Goat	Santa Cruz Biotec, Santa Cruz, CA.	Polyclonal anti DQ β-chain	Western Blot
WR18	Mouse	Serotec, Oxford, UK.	Pan HLA class II	T cell purification
TDR31.1	Mouse	Ancell, Bayport NJ.	Pan HLA class II	ELISA

2.1.4 Cell lines

A panel of EBV-transformed human B-Lymphoblastoid cell lines (B-LCLs) derived from HLA class II homozygous individuals were available as frozen stocks in the

laboratory of my supervisor, Dr. D.M. Altmann. Others were the generous gift of Dr. G. Lombardi, Department of Immunology, Imperial College London. The lines used in these experiments are detailed in Table 4. These cell lines were cultured in vented 75cm^2 flasks and divided every five days.

Bare Lymphocyte Syndrome (BLS) cell lines stably transfected to express several HLA-DQ dimers; endogenous HLA-DQ genes being transcriptionally silent, were the generous gift of Dr. W. Kwok Virginia Mason Research Center, Seattle, USA (Ettinger, Liu *et al.* 1998). Briefly, these cells lines are produced by retroviral mediated gene transfer. *DQA* and *DQB* cDNAs are cloned into separate retroviral vectors that are then used to transfect fibroblasts by calcium – phosphate precipitation. By co-culture of infected fibroblasts with BLS cells and subsequent culture under selection with G418 and hygromycin, stable transfectants expressing both α-and β-chains are generated and then maintained under selection with G418 (Sigma, UK) at 500μg/ml. To further maintain and improve level of HLA-DQ expression these cell lines were periodically FACS sorted to select out cells with high levels of expression.

An untransfected EBV transformed BLS cell line (HLA class II transactivator deficient) was used as a negative control throughout the study.

Table 2.2. EBV transformed B cell lines used in this study.

B Lymphoblastoid Cell Lines				
Cell Line	***DRB1****	***DQA1****	***DQB1****	**Serotype**
MOU (MANN)	*07011*	*0201*	*0202*	DR7 DQ2
VAVY	*0301*	*05011*	*0201*	DR3 DQ2
WT49	*03011*	*0201*	*0502*	DR3 DQ5
HOM2	*0101*	*0101*	*0501*	DR1 DQ5
LWAGS	*0102*	*0101*	*0501*	DR1 DQ5
BEC11		*0101*	*0503*	DR14 DQ5
AZB	*16011*	*01021*	*0502*	DR16 DQ5
TOK	*1502*	*0103*	*0601*	DR15 DQ6
PGF	*15011*	*01021*	*0602*	DR15 DQ6
SCHU	*1501*	*01021*	*0602*	DR15 DQ6
WT46	*1302*	*01021*	*0604*	DR13 DQ6
HOR	*1302*	*0102*	*0604*	DR13 DQ6
IDF	*11*	*0501*	*0301*	DR11 DQ7
TISI	*1103*	*0505*	*0301*	DR11 DQ7
SWEIG	*1101*	*0505*	*0301*	DR11 DQ7
600SF	*4**	*0301*	*0302*	DR4 DQ8
WT51	*04011*	*0301*	*0302*	DR4 DQ8
BOLETH	*0401*	*0301*	*0302*	DR4 DQ8
PRIESS	*0401*	*03*	*0302*	DR4 DQ8
Bare Lymphocyte Syndrome Cell Transfectants				
BLS DQ0302		*0501*	*0302*	
BLS DQ0602		*0102*	*0602*	
BLS DQ0604		*0102*	*0604*	
Other class II negative cell lines				
BLS	Class II transactivator deficient; express HLA class I			

*defined as *DRB1*0402* during this study

2.2 Methods

2.2.1 Synthesis of recombinant SPEA

Transformed *E. coli* BL21 expressing histidine tagged, full length *spea-1* were donated by Dr. S. Sriskandan (Department of Infectious Diseases, Imperial College, London) These were originally generated using pET19b expression system (Novagen, Madison, WI) (Sriskandan, Moyes *et al.* 1996). 50µl from glycerine stocks was inoculated into 100ml of Luria Bertrani (LB) broth (1% bacto-tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5mM KCl, pH7.0) containing ampicillin (200µg/ml) and incubated with shaking overnight at 37°C. The following morning 50mls of broth was made up to 250ml with fresh LB medium and ampicillin and reincubated for approximately 3 hours, until the optical density reached 0.5 (measured at 600nM). Keeping back 5mls of broth on ice as a control, isopropyl thio β-D galactoside (IPTG) (0.4mM) was added to the remainder to induce *spea* expression. Following 3 hours further incubation bacteria were harvested from the broth by centrifugation at 5000g for 5 min at 4°C. Samples of induced and uninduced bacteria were sonicated, boiled in SDS running buffer containing mercapto-ethanol and run on protein gel electrophoresis to confirm the presence of a band at 29kD in the induced cells (representing SPEA) absent from the uninduced cells. Recombinant SPEA plus the 10 histidine residue tag was purified using a nickel affinity purification column (HisBind Resin and HisBind buffer kit, Novagen, WI) following the manufacturers instructions. Bacterial pellets were sonicated in ice cold binding buffer and then centrifuged at 10000g for 20min. The pellets were then incubated in binding buffer (containing 6M urea) for 1 hour at 4°C to solubilise the recombinant SPEA.

Following further centrifugation the supernatant containing dissolved intracellular protein was filter sterilised and passed over a nickel loaded hisbind resin column. Bound histag-SPEA was eluted using 1M imidazole elute buffer containing 6M urea. The eluate was then dialysed into PBS at gradually reducing urea concentration over 5 days to allow refolding of the recombinant protein. After two final periods of dialysis against PBS without urea, protein concentration was measured using BCA-200 kit, (Pierce, IL), and purity assessed by SDS-PAGE. Recombinant SPEA produced by this protocol was then stored at –20°C in aliquots.

2.2.2 SDS Polyacrylamide gel-electrophoresis and western blotting

Polyacrylamide gels of required concentrations were cast using Protogel regents (National Diagnostics, Hull, UK) in Hoefer™ gel casting units (Amersham Pharmacia Biotech, Bucks, UK). Appropriate volumes of sample were denatured in 1x SDS sample buffer containing 1mM dithioreitrol (DTT) and run at 20mA in Hoefer electrophoresis units containing running buffer. To visualise protein bands gels were stained with Coumassie Blue stain, followed by destaining solution for 2-12 hours.

For Western blots, proteins were transferred onto Hybond P membranes (Pharmacia Biotech, Bucks, UK) at 70V, 0.18mA for 1 hour at 4°C in a Hoefer TE 22 Transphor electrophoresis unit (Amersham Pharmacia Biotech, Bucks, UK). Membranes were stained with Ponceau S stain to check for transfer and then blocked overnight with 5% skimmed milk powder in PBS overnight at 4°C. Membranes were then probed with primary antibody for 1 hour at room temperature on a rotary shaker, washed six times with 0.1% tween 20 in PBS, and probed with secondary biotin conjugated

secondary antibody for 1 hour at room temperature. After further washing detection was with streptavidin HRP (Serotec Ltd, Oxford, UK) 1:5000 for 1 hour on a rotary shaker. After further washing blots were developed using ECL chemiluminescent kit (Amersham Int, Little Chalfont, UK) according to manufacturers instructions.

2.2.3 Preparation of human peripheral blood mononuclear cells

20-50ml of blood was drawn from healthy donors and diluted 1:2 in RPMI. 25ml aliquots of diluted blood were overlayed on an equal volume of Ficoll-Paque (Pharmacia Biotech) and centrifuged at 800g for 35 minutes. The buffy interface was removed using a sterile Pasteur pipette and washed twice in RPMI for 10 minutes. Cells were then counted using a haemocytometer and resuspended ready for use in complete RPMI.

2.2.4 Preparation of murine splenocytes.

Spleens were dissected out of freshly sacrificed mice and immersed in complete RPMI containing $5x10^{-5}M$ 2-mercaptoethanol. By repeatedly passaging medium through the spleen tissue, using a fine bore needle and syringe, until blanched, approximately $1x10^7$ splenocytes were obtained per spleen. Cells were then washed, counted by haemocytometer and resuspended in complete RPMI plus 2ME ready for use.

2.2.5 Proliferation assays

In experiments comparing stimulation by different superantigens, human PBMCs ($2x10^5$ cells/well) in 200µl RPMI-1640 supplemented with 10% fetal calf serum, 2mM glutamine, 50U/ml penicillin and 50µg/ml streptomycin (Life Technologies,

Paisley, UK) were stimulated in a 96 well flat bottomed plate (Corning Costar, UK). Each well was repeated in triplicate. Stimulus was either bacterial superantigen, negative control (media alone) or positive control (phytohaemagglutinin). Plates were incubated for 48 hours in the presence of stimulus at 37°C, 5%CO_2. After 48 hours 1μCi methyl-^{3}thymidine was added to each well. After a further 16 hours incubation, cells were harvested on to filter mats in a Betaplate™ 96 well harvester (Wallac and Berthold, Milton Keynes, UK.). Counts per minute were measured in a Betaplate™ Liquid Scintillation Counter (Wallac and Berthold, Milton Keynes, UK.).

2.2.6 Culture of B cell lines

EBV transformed B-LCLs and BLS HLA transfectants were all cultured in 75cm2 vented tissue culture flasks at 37°C in a humidified atmosphere of 95% air, 5% CO_2 in complete RPMI.

2.2.7 Flow cytometry

Cells, whether purified PBMCs, T cells, mouse splenocytes or cultured lines were washed in FACS buffer, resuspended at $5x10^6$ cells ml and then aliquoted into wells of a U-bottomed 96 well plate. Cells were then spun down by centrifugation at 1200rpm for 2minutes, buffer poured off and pellets resuspended. Antibodies were then added either neat, 1-5μl per pellet depending on the antibody, or made up to 20μl in FACS buffer. Plates were incubated for 40 minutes at 4°C and intermittently agitated to ensure even antibody staining of cells. Cells were washed three times in 250μl of FACS buffer between 1st and 2nd layer staining and prior to final analysis. For analysis cells were resuspended in 400μl of FACS buffer and analysed in a

FACSCalibur flow cytometer (Becton Dickenson UK Ltd, Oxford, UK). 5000 - 20000 gated events were collected for analysis depending on cell type.

2.2.8 Cytokine quantification

Human and murine cytokines were measured by ELISA using paired antibodies purchased from R&D systems Ltd according to the manufacturer's instructions.

2.2.9 Mouse methods

Female C57/BL6 mice (Charles River, UK), and HLA-DQ3 AbO transgenic mice (Dr. D. Altmann, Human Disease Immunogenetics Group, Imperial College, London) weighing 25-30g in *in vivo* experiments analysing the effect of superantigen exposure on Vβ repertoire. BALB/c (Charles River UK) and DO11.10 (Dr. D. Altmann, Human Disease Immunogenetics Group, Imperial College, London) mice were used to harvest spleens for proliferation assays.

All animals received food and water *ad libitum* during *in vivo* experiments. All procedures were conducted within local and Home Office guidelines.

2.2.10 Statistics

Student t-test was used for comparison of parametric data from *in vitro* experiments. Values of $P<0.05$ were considered significant. Analysis was performed using SigmaStat software, SPSS Inc, Chicago IL.

CHAPTER 3

Influence of HLA class II polymorphism on superantigen binding to B cell lines

3.1 Introduction

Several lines of evidence indicate that allelic HLA class II polymorphisms are likely to result in differences in the binding of superantigens. Interspecies and interisotype differences in superantigen binding by MHC class II are well established. Superantigen mutagenesis studies indicate that the affinity of superantigen – class II binding determines potency in T cell proliferation (Kline and Collins 1996). Crystal structure and mutagenesis studies of the class II – superantigen interaction have demonstrated that two superantigen binding sites exist on the HLA class II molecule. One is a low affinity site on the α-chain (kD ~10^5M) and the other a high affinity site on the β-chain (kD~10^7M) (Papageorgiou and Acharya 1997; Li, Llera *et al.* 1999). Both binding sites have now been defined by X-ray crystallography. The low affinity α-chain site in the complex between SEB and HLA-DR1 (Jardetzky, Brown *et al.* 1994) and the high affinity β-chain site in the complexes between SPEC and HLA-DR1 (Li, Li *et al.* 2001) and SEH and HLA-DR1 (Petersson, Hakansson *et al.* 2001). These studies demonstrate that the α chain site, while non-polymorphic in HLA-DR, is polymorphic in HLA-DQ (Sundberg and Jardetzky 1999) and that the β-chain site lies in a polymorphic region both in DR and DQ.

Previous assessments of differences in superantigen binding by different class II molecules have principally been conducted using cell lines transfected to express particular HLA class II molecules. A common approach has been to use HLA class II transfectants of mouse L cell (embryonic fibroblast) lines. There are a number of limitations to this approach. First and principally, the level of class II expression on transfected cell lines tends to be low and requires selective culture conditions and FACS sorting of cells for maintenance of expression. Second, because transfectants lack the intracellular processing pathways for normal class II assembly, peptide loading and chaperoning to the cell surface, the class II expressed at the cell surface of transfected cell lines may differ from the class II expressed by professional antigen presenting cells in terms of superantigen binding and presentation (Lavoie, Thibodeau *et al.* 1997; Hogan, VanBeek *et al.* 2001). Such limitations may explain the failure of previous studies using such approaches to detect an effect of HLA polymorphisms on the binding and presentation of superantigens (Herman, Croteau *et al.* 1990; Scholl, Diez *et al.* 1990). A third limitation on this approach arises because of HLA class II diversity itself. With over 650 HLA class II alleles currently described (Robinson, Waller *et al.* 2003), it is simply impractical to generate a panel of comparable transfectants to screen even the more common HLA class II alleles for differences in superantigen binding.

HLA class II homozygous, EBV transformed, B lymphoblastoid cell lines (B-LCLs), unlike HLA class II transfected cell lines, offer stable and high levels of class II expression. The class II expressed on B-LCLs has been through all the normal intracellular processing of heterodimer formation, peptide loading and transport to the

cell surface. Additionally, unlike class II transfected cell lines, B-LCLs have normal expression of co-receptor factors such as CD28 which are important in superantigen presentation to T cells (Lando, Olsson *et al.* 1996; Saha, Harlan *et al.* 1996; Rajagopalan, Smart *et al.* 2002). One potential limitation on the use of B-LCLs in this context arises from the fact that many bacterial superantigens, principally the staphylococcal enterotoxins utilise both HLA-DR and HLA-DQ binding to achieve T cell activation. However, some superantigens do appear to be restricted to one class II isotype. SPEA and SSA for example preferentially utilise HLA-DQ (Imanishi, Igarashi *et al.* 1992; Norrby-Teglund, Nepom *et al.* 2002).

The experiments described in this chapter exploit the HLA-DQ restriction of SPEA to allow use of B-LCLs to screen a range of DQ alleles for differences in SPEA binding. HLA-DQ transfected Bare Lymphocyte Syndrome (BLS) cell lines are then used to study binding of SPEA to selected HLA-DQ alleles. It is demonstrated that HLA-DQ alleles in which the SPEA-binding α-chain is *HLA-DQA1*01* show greater binding of SPEA than HLA-DQ*A1*03* or *DQA1*05*. By making similar comparisons of B-LCL binding of the staphylococcal superantigen SEA, it is also demonstrated that binding of this superantigen is greater to *HLA-DRB1*0401* and *DR15* than to *HLA-DRB1*11*. Analysis of the amino acid sequences of the DQ α-chain and the DR β-chain at sites of superantigen binding reveals that polymorphisms differentiating these alleles are likely to be responsible for the observed differences in binding.

3.2 Methods

3.2.1 Biotinylation of recombinant SPEA

Recombinant SPEA (rSPEA) was biotinylated using EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit (Pierce, IL). SPEA in PBS was dialysed for 24 hours against tris buffer (Tris-HCl 20mM NaCl 150mM pH8). Sulpho-NHS-LC biotin was dissolved in Tris-buffer to a concentration of 10mg/ml and the pH adjusted back to 8.0. Biotin solution was added to the solution of SPEA dropwise to achieve a 20 Molar excess of biotin to rSPEA. After 2 hours incubation on a magnetic stirrer at 4°C the volume was made up to 5mls with tris buffer and the biotinylated protein was separated from free biotin by High Pressure Liquid Chromatography (HPLC). Protein containing eluates from the HPLC were concentrated using an Amicon stir cell, PM20 membrane (Amicon, MA). The final protein concentration was measured using BCA-200 kit, (Pierce IL), and biotinylation was confirmed by ELISA as follows.

The wells of a 96 well Maxisorp C-bottom ELISA plate (Nunc, UK) were coated with 100µl/well of biotinylated SPEA in five serial 1:10 dilutions, and incubated for 1 hour at 37°C. Control wells were coated with serial dilutions of antimouse Ig-biotin (Jackson, PA) in the same way. After 5 washes, 100µl/well of avidin-peroxidase (Jackson, PA), 1:1000 in PBS, 10%BSA was added to each well and incubated for 15 minutes at room temperature. After 5 further washes the ELISA was developed with Tetramethyl benzidine (TMB) substrate (Sigma, UK), and the reaction stopped after 1 minute with 1M H_2SO_4. A colour change at dilutions equal to, or greater than, the greatest dilution of positive control which produced a colour change was considered to indicate satisfactory biotinylation of SPEA.

3.2.2 Flow Cytometric Analysis of Superantigen Binding

Binding of biotinylated SPEA to untransfected BLS cells, B-lymphoblastoid cells and HLA-DQ transfectants of BLS cells was demonstrated as follows. Cultured cells were harvested and washed twice in cold FACS buffer (PBS supplemented with 1% fetal calf serum and 0.01% sodium azide). $5x10^5$ cells were incubated with various concentrations of biotinylated SPEA for 1 hour at 4°C in U bottomed 96 well plates on a rotary shaker. Cells were then washed twice prior to incubation for 1 hour under the same conditions with the secondary reagent Extra-avidin-PE (Sigma, UK). After two further washes cells were resuspended in 500µl of FACS buffer and analysed by flow cytometry (Facscalibur Becton-Dickenson, Cellquest software).

Level of HLA-DQ expression on each cell line was measured in each experiment using mouse monoclonal antibodies to HLA-DQ and a donkey anti-mouse-FITC second layer (Jackson, PA).

Binding of biotinylated SPEA to the cells was measured from flow cytometry data as follows. 20 000 cells falling within a healthy lymphocyte gate were collected. Mean fluorescence intensity (MFI) of cells incubated with biotinylated SPEA and streptavidin-PE was divided by MFI of cells incubated with unbiotinylated SPEA and streptavidin-PE. Expression of HLA-DQ on the cell surface was measured using a panel of anti-DQ antibodies each recognising a different monomorphic determinant; L2 (Fermand, Chevalier *et al.* 1986), Leu10 (Becton-Dickenson UK) or SPV-L3 (Spits, Borst *et al.* 1984). The MFI for cells incubated with each anti-DQ and anti-mouse-FITC was divided by MFI for cells incubated with isotype control and anti-mouse-FITC and the resulting fluorescence shift then expressed as a percentage of the

highest fluorescence shift of any cell line with that antibody. The mean of the percentage shift obtained for each antibody was taken as a measure of the relative DQ expression of a particular cell line. In order to correct binding of SPEA to cells for differences in the level of HLA-DQ expression, the fluorescent shift for SPEA binding was divided by the level of HLA-DQ expression and multiplied by 100, for ease of presentation.

Binding of SEA was measured in exactly the same way. DR expression in these experiments was determined by binding of the antibody L243 (Lampson and Levy 1980) which binds the non-polymorphic DR α-chain.

3.3 Results

3.3.1 Characterisation of biotinylated SPEA

Recombinant SPEA was shown by SDS-PAGE to run as a single band at a molecular weight of around 29kD, corresponding to the predicted weight of SPEA-histag. Following biotinylation, although some protein was lost during purification the biotinylated protein ran as a single band at the same molecular weight on SDS-PAGE (Fig. 3.1).

As demonstrated previously by Dr. Sriskandan (Department of Infectious Diseases, Imperial College, London personal communication), rSPEA has approximately half the mitogenic activity of purified SPEA. This difference is likely to result from contamination of purified SPEA by other more potent superantigens such as Streptococcal Mitogenic Exotoxin Z (SMEZ) rather than recombinant SPEA being of lower mitogenic potency than naturally occurring SPEA. The process of biotinylation of recombinant SPEA was not associated with any loss of mitogenicity (Fig. 3.2).

In order to confirm that the specific superantigenic activity of recombinant SPEA was unaffected by biotinylation, the Vβ specific change in T cell repertoire, following biotinylated SPEA stimulation of PBMCs was assessed. In accordance with the established Vβ specific response to SPEA, a greater than five fold expansion of Vβ14 and Vβ12 T cells was observed in response to 100μg/ml biotinylated SPEA with a corresponding reduction of other Vβs (Fig. 3.3).

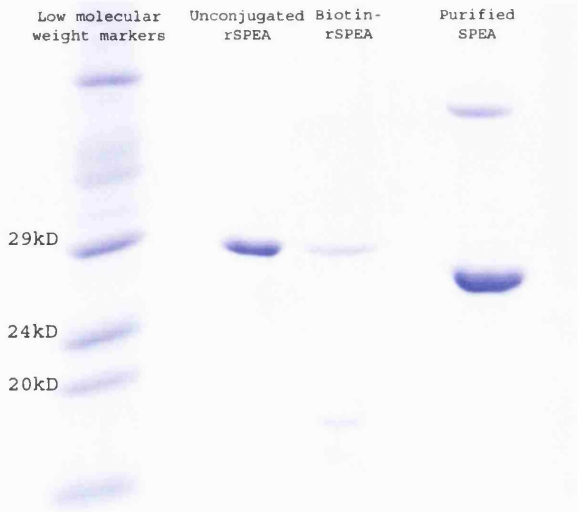


Fig. 3.1. SDS-PAGE of different SPEA preparations. Recombinant SPEA and biotinylated recombinant SPEA are shown and compared with molecular weight markers and purified SPEA.

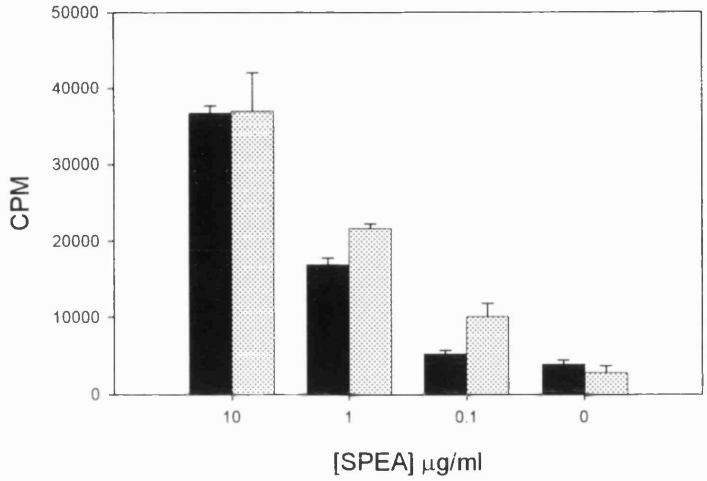


Fig. 3.2. Mitogenicity of unconjugated and biotinylated SPEA. PBMC proliferation in response to unconjugated (■) and biotinylated SPEA (■). Histogram bars show mean responses from triplicate culture (+/-SD) using cells from a single donor.

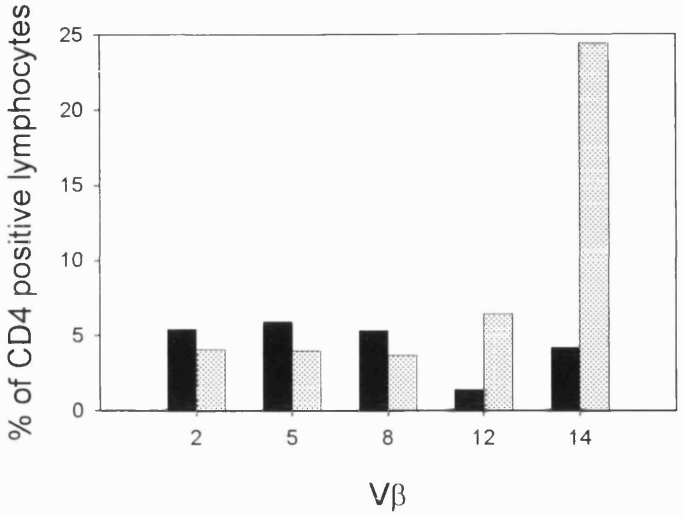


Fig. 3.3. Vβ specific T cell repertoire changes following stimulation with unconjugated and biotinylated SPEA. Percentages of total CD4 positive lymphocyte population from a single donor are shown for five TCR Vβ types.

3.3.2 Validation of SPEA binding to EBV transformed B cell lines

SPEA binding to HLA-DQ expressing cell lines was measured as described above. By way of illustration, fluorescence histograms of SPEA binding to two B-LCLs are shown in Figure 3.4.

The contribution of HLA-DQ binding to the observed total cell bound SPEA was assessed using saturating concentrations of anti-DQ and anti-DR antibodies. The antibody L243, which binds the DR α-chain, competed with SPEA for binding to B cells to only a minimal extent. In contrast, the antibody L2, which binds the DQ α-chain, produced near 100% blocking of SPEA binding sites at saturating concentrations of antibody. Similarly in the presence of fifty fold excess unbiotinylated SPEA, blocking of the biotinylated toxin was around 80% (Fig. 3.5).

3.3.3 Assessment of level of DQ expression on B cell lines

Since both chains of the HLA-DQ molecule are polymorphic, monoclonal antibodies recognising monomorphic determinants may have varying affinities for different-DQ molecules. Given this fact and the possibility that given cell lines may modulate class II expression depending on culture conditions, considerable attention was paid to the need obtain a true relative value for DQ expression for each cell line at the time of each experiment. This was ultimately achieved by staining cells with a pooled panel of monoclonal antibodies each recognising a different monomorphic determinant; Leu10, Tü169, SPVL-3 and L2. Each antibody was assessed at a range of dilutions to determine the concentration required to saturate binding. Cell lines expressing HLA-

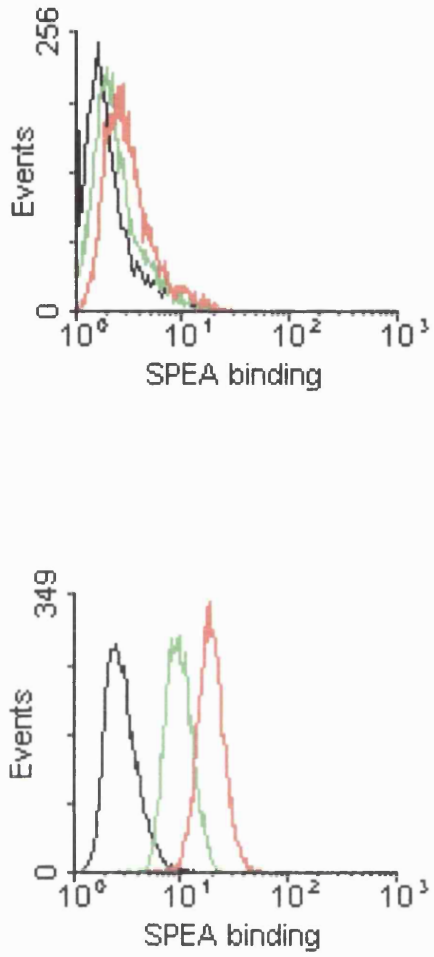


Fig. 3.4. Binding of SPEA to high and low binding B-LCLs. Two cell lines are shown for illustration. **A)** TISI (*DQA1*0501*/DQB1*0302) and **B)** PGF (*DQA1*0102/DQB1*0602*). In each case 10µg (red), 5µg (green) and negative control (black) are shown.

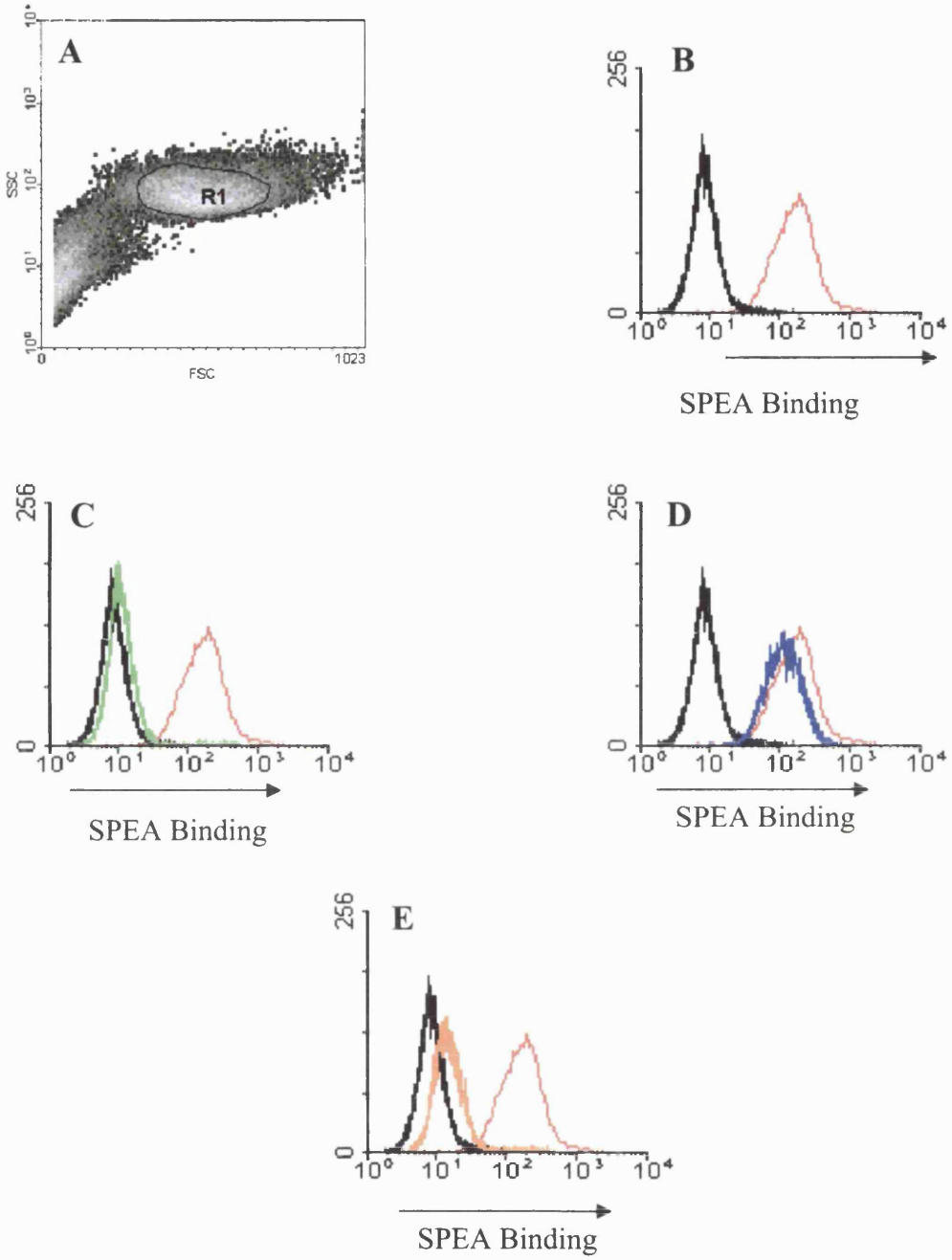


Fig. 3.5. Specificity of SPEA binding to B-LCLs. A, Forward vs. Side Scatter plot showing cells gated on a healthy lymphocyte population. **B**, Biot - SPEA 10mg/ml (——) and 0mg/ml (——). **C**, effect of preincubation in presence of L2 (anti DQα) (——). **D**, effect of preincubation in presence of L243 (anti DRα) (——). **E**, effect of co-incubation in presence of 50 fold excess unbiotinylated SPEA (——).

DQ2 were found to bind all antibodies except Tü169 poorly, presumably due to the poor affinity of the other antibodies for HLA-DQ2. Consequently we excluded HLA-DQ2 from any further analysis. Cell lines expressing HLA-DQw1 or HLA-DQw3 were analysed for their binding of Leu10, L2 and SPV-L3 by flow cytometry, using isotype matched controls. The binding of each antibody by each cell line was then expressed as a percentage of the highest. This generated three percentage values for each cell line, one for each of the three antibodies. The mean of these three percentage values was taken as a measure of the relative level of HLA-DQ expression. To account for variations in level of class II expression this analysis was performed in each experiment. Figure 3.6 shows the results of one such comparison of B-LCL DQ expression. For each cell line the mean of the three antibody binding results is shown +/- 1SD. It is noteworthy that the standard deviations are small in comparison with the differences between cell lines, indicating consistency between the three antibodies. Furthermore, differences between cell lines of the same HLA-DQ genotype are wide, while the ranges of level of expression measured for the two serotypes DQw1 and DQw3 overlap extensively. This indicates that the differences observed in antibody binding represent differences in DQ expression rather than differences in antibody affinity.

3.3.4 SPEA binding to HLA homozygous B-LCLs

If all HLA-DQ molecules were equal in their binding of SPEA, SPEA binding to B cell lines would be directly related to level of HLA-DQ expression. However, in multiple correlations of SPEA binding with level of DQ expression on a panel of cell lines expressing common HLA-DQ molecules, we found that HLA-DQ genotype

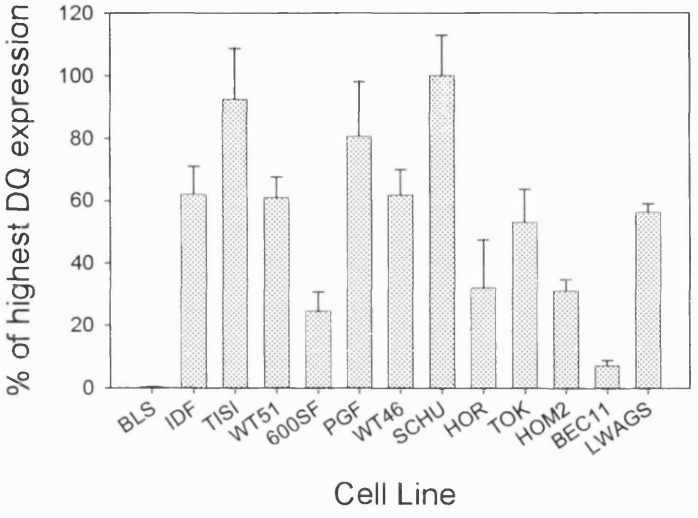


Fig. 3.6. Assessment of level of DQ expression on B-LCLs. For each cell line the mean percentage of highest MFI for each DQ antibody is shown. Error bars show one standard deviation.

markedly influenced SPEA binding. While for cell lines expressing *HLA-DQA1*01* α chains, the amount of SPEA binding detected at a given concentration of SPEA did indeed correlate closely with the level of HLA-DQ expression, SPEA binding to cell lines expressing *HLA-DQA1*03* or *05* α-chains was minimal irrespective of level of DQ expression. In order to allow statistical comparison of SPEA binding between DQ*A1*01* and DQ*A1*03/05* expressing cell lines, a calculation of SPEA binding corrected for level of DQ expression was performed. Statistical comparison was then made by unpaired t-test using SigmaStat software. Three illustrative experiments, conducted using concentrations of 5μg/ml and 10μg/ml are shown in Figure 3.7a, b & c.

Because variations in cell cycle and culture conditions can lead to fluctuations in the level of cell surface HLA expression, we then analysed the relationship between HLA-DQ expression and SPEA binding in several independent cultures of *DQA1*01* lines (PGF and WT46) or *DQA1*05* lines (IDF and TISI). For each of the *DQA1*01* cell lines a correlation was observed between DQ expression and SPEA binding while despite higher levels of DQ expression SPEA binding to the two *DQA1*05* cell lines was minimal and unrelated to level of DQ expression (Fig 3.8).

3.3.5 SPEA binding to HLA-DQ transfected BLS cell lines

In order to confirm positively that the differences in SPEA binding observed between B-LCLs expressing *HLA-DQA1*01* and *HLA-DQA1*03* or **05* could not be attributed to differences in expression of other cell surface determinants such as HLA-DR, several HLA-DQ transfectants of a bare lymphocyte syndrome cell line were employed. The cell lines used were all the gift of Dr. W. Kwok (Virginia Mason

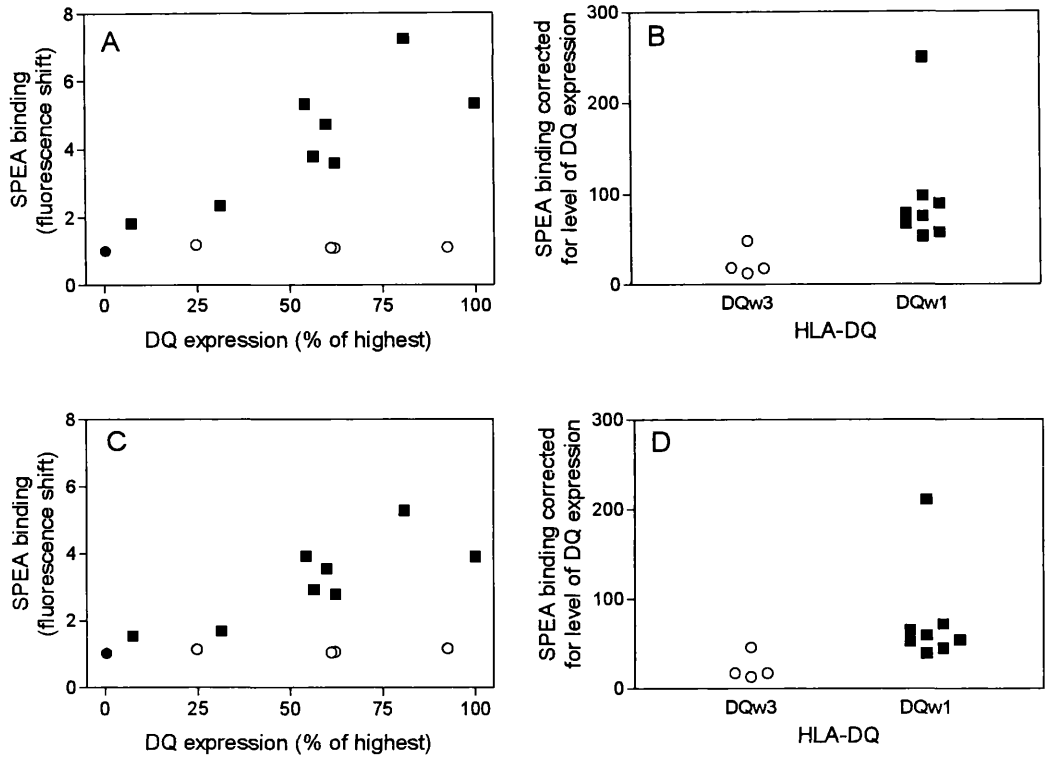


Fig. 3.7a. Binding of biotinylated SPEA to a panel of HLA homozygous B lymphoblastoid cell lines. A & B SPEA 10μg/ml, **C & D**, SPEA 5μg/ml. Cell lines expressing *HLA-DQA1*01* (DQw1 serogroup) (■), cell lines expressing either *HLA-DQA1*03* or **05* (DQw3 serogroup) (○), class II negative cell line (●). Statistical comparison of SPEA binding corrected for level of DQ expression by t-test, **B**; DQw1>DQw3 P=0.003, **D**; DQw1>DQw3 P=0.016.

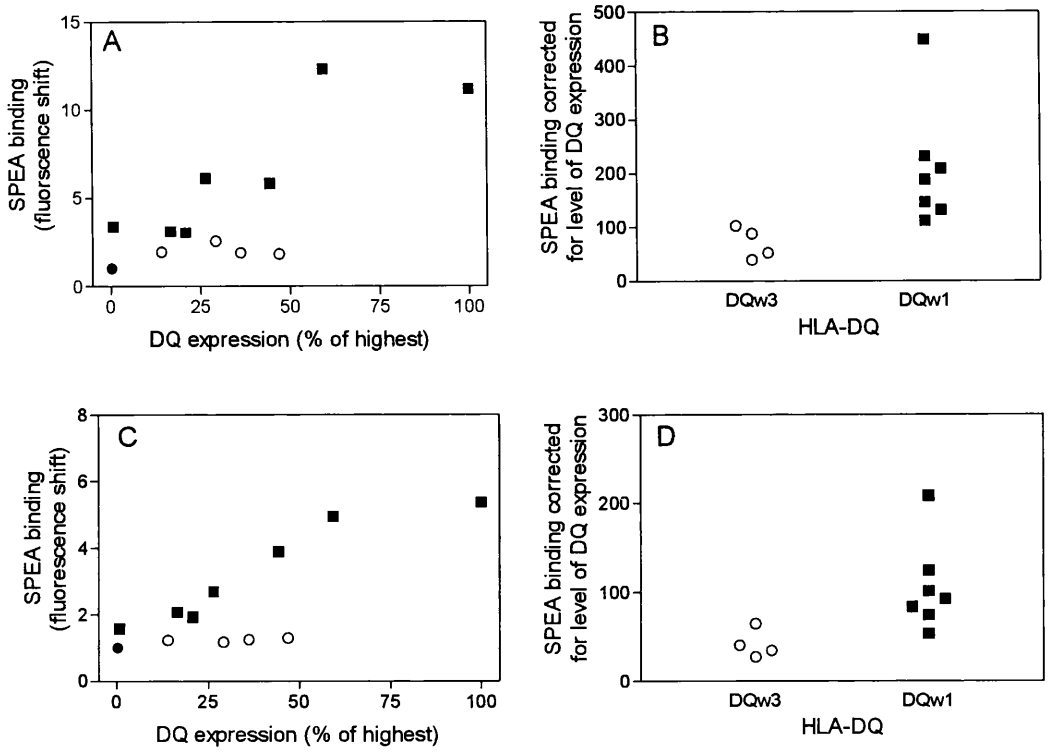


Fig. 3.7b. Binding of biotinylated SPEA to a panel of HLA homozygous B lymphoblastoid cell lines. A & B SPEA 10μg/ml, **C & D**, SPEA 5μg/ml. Cell lines expressing *HLA-DQA1*01* (DQw1 serogroup) (■), cell lines expressing either *HLA-DQA1*03* or **05* (DQw3 serogroup) (○), class II negative cell line (●). Statistical comparison of SPEA binding corrected for level of DQ expression by t-test, **B**; DQw1>DQw3 P=0.006, **D**; DQw1>DQw3 P=0.012.

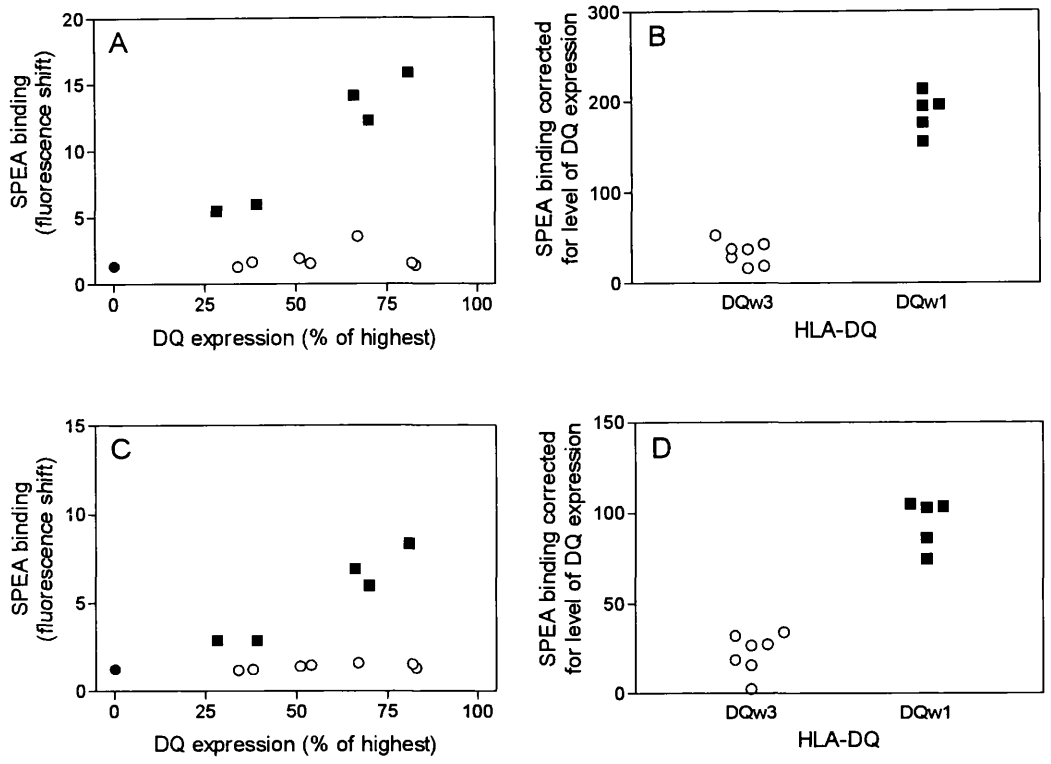


Fig. 3.7c. Binding of biotinylated SPEA to a panel of HLA homozygous B lymphoblastoid cell lines. **A** & **B** SPEA 10μg/ml, **C** & **D**, SPEA 5μg/ml. Cell lines expressing *HLA-DQA1*01* (DQ6 serogroup) (■), cell lines expressing either *HLA-DQA1*0301/DQB1*0301* (DQ7 serogroup) (○), class II negative cell line (●). Statistical comparison of SPEA binding corrected for level of DQ expression by t-test, **B**; DQ6>DQ7 P=0.001, **D**; DQ6w1>DQ7 P=0.001.

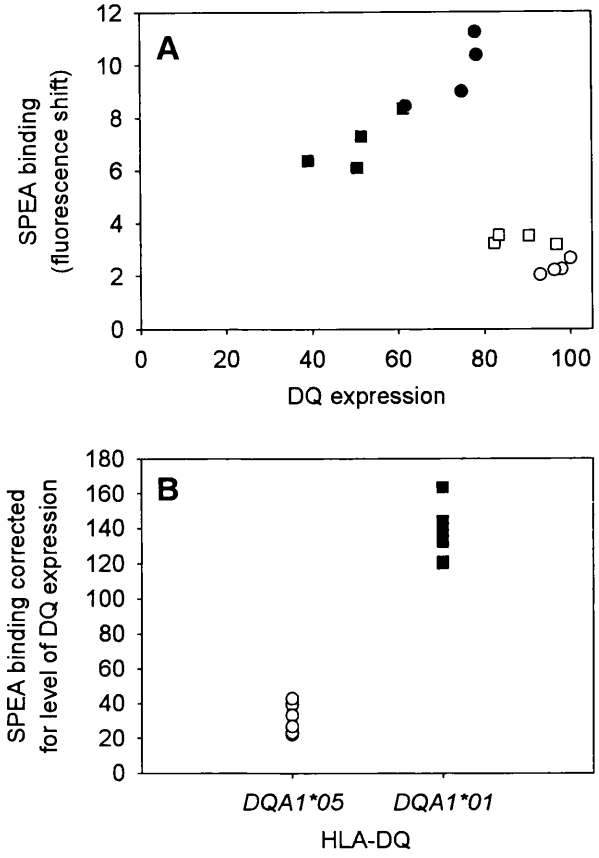


Fig. 3.8. Influence of HLA-DQ expression on SPEA binding. A) Four data points are shown for each of four cell lines. PGF (●) and WT46 (■), both *HLA-DQA1*01* and IDF (□) and TISI (○), both *HLA-DQA1*05*. Each point represents an independent cell culture. **B)** Comparison of SPEA binding corrected for level of DQ expression, *HLA-DQA1*01* (■) *vs HLA-DQA1*05* (○) $P<0.001$ by t-test.

Research Centre, Seattle, WA) and have been described previously (Kwok, Kovats *et al.* 1993). The parent cell line, an EBV transformed B cell line derived from an individual with type II Bare Lymphocyte Syndrome was used as a control. The three cell lines used were *DQA1*0301/DQB1*0302*, *DQA1*0102/DQB1*0602* and *DQA1*0102/ DQB1*0604* transfectants. Although levels of DQ expression on these cell lines were low in comparison with B-LCLs, the same relationship between DQ expression and SPEA binding was observed. Specifically, both the cell lines expressing *DQA1*01* bound higher levels of SPEA corrected for level of DQ expression than did the *DQA1*03* expressing cell line. In order to make statistically valid comparisons of these three cell lines, multiple independent cultures of each were set up and analysed. In figure 3.9 two experiments are shown. In the first, three cultures of the *DQA1*0102/DQB1*0602* transfectant are compared with four of the *DQA1*0301/DQB1*0302* transfectant. In the second, five cultures of the *DQA1*0102/DQB1*0604* transfectant are compared with eight of the *DQA1*0301/DQB1*0302* transfectant.

3.3.6 SEA binding to HLA homozygous B-LCLs

To determine whether HLA class II polymorphisms might determine the binding of other superantigens we chose next to study the staphylococcal superantigen SEA. SEA exemplifies a different mode of superantigen class II interaction since it binds predominantly to HLA-DR and has two binding sites, one a high affinity site for the DR β-chain and one a low affinity site for the α-chain. While *HLA-DRA1* is non-polymorphic, *HLA-DRB1* is considerably more polymorphic than *HLA-DQA1.* Consequently, in making comparisons between different *HLA-DRB1* alleles using the

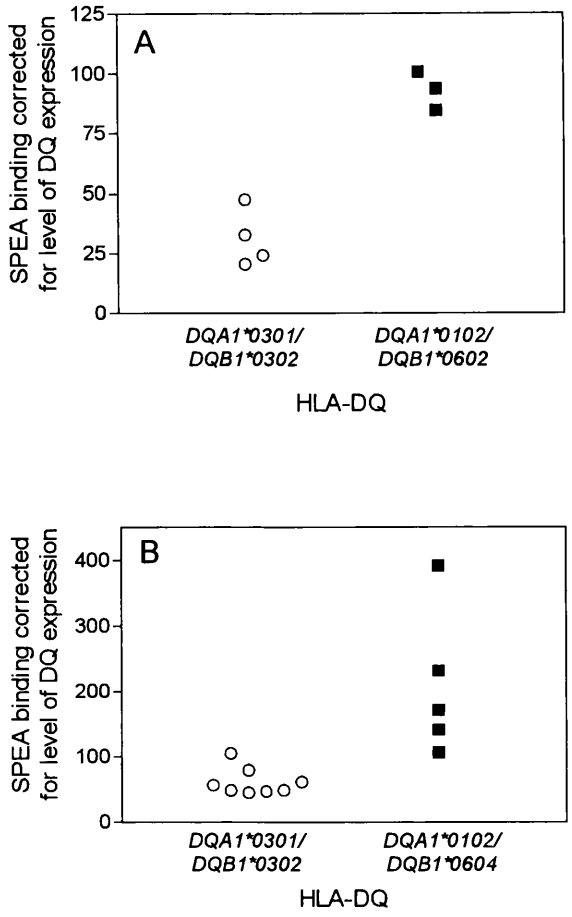


Fig. 3.9. Binding of SPEA to HLA-DQ transfected BLS cell lines. Binding of SPEA at 10μg/ml is shown. **A;** replicates of a *DQA1*0301 / DQB1*0302* transfectant (o) and a *DQA1*0102 / DQB1*0602* transfectant (■), P<0.001 by t-test, **B**; replicates of a *DQA1*0301 / DQB1*0302* transfectant (o) and a DQA1*0102 / DQB1*0604 transfectant (■), P=0.0035 by t-test.

B-LCL panel we chose to analyse SEA binding only to those DR types for which we had two or more *HLA-DRB1* identical cell lines, namely DR11 (IDF and TISI), DR4 (600sf and WT51) and DR15 (PGF, TOK, SCHU). Making comparison of SEA binding to the two DR4 cell lines and the two DR11 cell lines both DR11 cell lines bound SEA less than the cell line WT51 (*DRB1*0401*), however the cell line 600sf (DR4, undetermined subtype) bound SEA comparably with the low binding DR11 cell lines (Fig. 3.10a). The cell line 600sf was subsequently *HLA-DRB1* genotyped, and found to be *DRB1*0402* homozygous. To determine whether the difference in SEA between WT51 and 600sf was the result of DR4 polymorphisms differentiating *DR0401* and *DR0402* and to confirm the differences between DR0401, DR15 and DR11, further B-LCLs were obtained; SWEIG (*DRB1*1103*) and BOLETH (*DRB1*0401*) both donated by Dr. G. Lombardi, Dept. of Immunology Imperial College, and PRIESS (*DRB1*0401*) donated by Dr Mark Peakman, Department of Immunology Guys, Kings and St. Thomas' Medical School. When these cell lines were analysed, statistically higher binding of SEA was observed to DR15 and DR0401 than to DR11. The single *DRB1*0402* cell line 600sf was again found to exhibit low binding of SEA similar to DR11 (Fig. 3.10b).

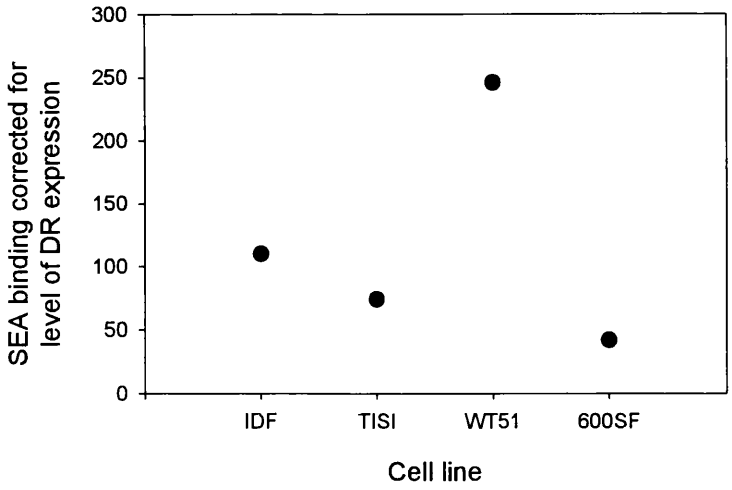


Fig. 3.10a. Binding of SEA to HLA homozygous B-LCLs. Binding of SEA (10μg/ml) to two DR11 cell lines (IDF and TISI) and two DR4 cell lines WT51(*DRB1*0401*) and 600sf (*DRB1*0402*) are shown.

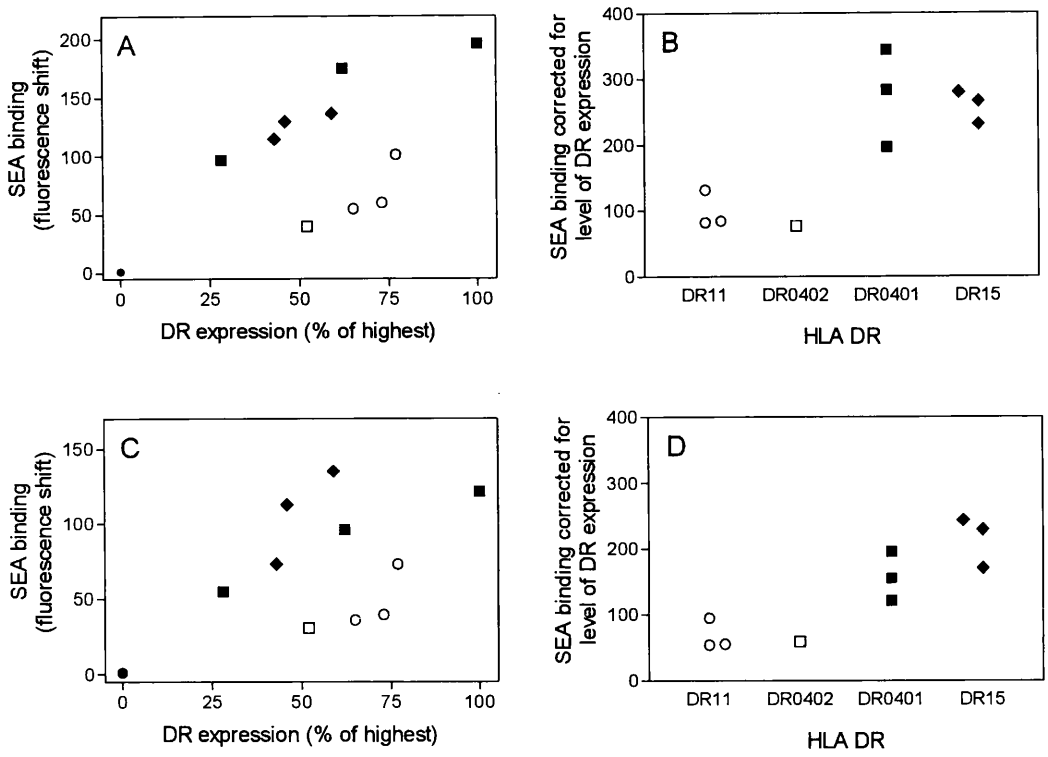


Fig. 3.10b. Binding of SEA to HLA homozygous B-LCLs. A & B; SEA 10μg/ml, **C & D**; SEA 5μg/ml. Cell lines expressing *HLA-DR0401* (WT51, BOLETH, PRIESS) (■), HLA-DR*0402* (600sf) (□), *HLA-DR1103* (IDF, TISI, SWEIG) (o), *HLA-DR15* (PGF, TOK, SCHU) (◆) and a class II negative cell line (BLS) (●) are shown. P values comparing binding to *DR0401* or *DR15* with *DR11* by t-test, **B**; *HLA-DR0401* P=0.019, *HLA-DR15* P=0.002, **D**; *HLA-DR0401* P=0.024, *HLA-DR15* P=0.005.

3.4 Discussion

Binding by MHC class II is a prerequisite for superantigen activation of T cells (Fleischer and Schrezenmeier 1988). Although interspecies and interisotype (Norrby-Teglund, Nepom *et al.* 2002) differences in binding and presentation of superantigens are established, the possibility that HLA class II isotype polymorphisms might influence binding of individual superantigens has not been studied extensively.

Chatila and co-workers failed to identify allelic differences in HLA class II binding of TSST-1 and SEB using transfected L cells and radioligand binding studies (Scholl, Diez *et al.* 1990). Two subsequent studies have found some minor allelic differences. In the first, Herman *et al* used HLA-DR transfected mouse L cells to assess binding and presentation to T cell hybridomas of a panel of staphylococcal superantigens (Herman, Croteau *et al.* 1990). The only differences in binding they identified related to poor binding of SEA and SEE by DRw53. This has subsequently been shown to relate to a single substitution of tyrosine in DRw53 for the otherwise highly conserved histadine residue at position 81 of the DR β-chain (Herman, Labrecque *et al.* 1991; Karp and Long 1992). Since DRw53 is co expressed with DR4, 7 and 9 this observation can not shed any light on interindividual differences in susceptibility to the effects of SEA or SEE. In the second report, Hargreaves *et al.* (Hargreaves, Brehm *et al.* 1995) identified a glycine residue at position 84 of the DR1 β-chain that is required for SEE presentation and in so doing observed that DQw2 supports SEE activation of T cells better than DQw6 (glutamine *vs* glutamic acid at position 84).

Over the last decade, the crystal structures of several superantigens have been published; SPEA (Papageorgiou, Collins *et al.* 1999), SEA (Schad, Zaitseva *et al.*

1995; Petersson, Thunnissen *et al.* 2002), SEB (Papageorgiou, Tranter *et al.* 1998), and SPEC (Roussel, Anderson *et al.* 1997). The mechanisms of superantigen binding to HLA class II have been elucidated through mutagenesis binding studies and the co-crystallisation of several superantigens, complexed with HLA class II (Jardetzky, Brown *et al.* 1994; Kim, Urban *et al.* 1994; Dessen, Lawrence *et al.* 1997; Li, Li *et al.* 2001; Petersson, Hakansson *et al.* 2001). For example, the co-crystal structure of SEB and DR1 has allowed the superantigen binding sites on the DR α-chain to be defined (Jardetzky, Brown *et al.* 1994).

The crystal structure of the streptococcal superantigen SSA has also been described (Sundberg and Jardetzky 1999). SSA has marked sequence homology with SEB and SPEA. Like both these superantigens SSA binds to the class II α-chain and like SPEA the affinity for DQ α-chain is markedly higher than DR α-chain. Sundberg and Jardezky used the SSA and SEB crystal structures to predict the DQ α-chain amino acids involved not only in SSA binding but SPEA binding also. They noted that these sites are polymorphic and concluded that it is unlikely that SSA, and other superantigens which bind the DQ α-chain, bind to all DQ alleles equally. Table 1 shows the amino acid sequence of the *DQA1*01* α-chain at sites implicated in superantigen binding. Analysis of the amino acid sequence of the DQ α-chain at these sites shows that the α-chains encoded by the subdivisions of *DQA1*01* are, with a single exception, non polymorphic at sites of superantigen binding (Marsh, Parham *et al.* 2000). The exception, *DQA1*0103,* contains one amino acid substitution, K41R, which is likely to have minimal structural impact since here one polar basic amino acid is substituted for another. In contrast the α-chains encoded by the subdivisions of

*DQA1*03* and *DQA1*05* all share three substitutions when compared with the *DQA1*01* α-chain; F61G, T64R, and I66M. Each of these substitutions are likely to be structurally significant. In the first, phenylalanine (hydrophobic, aromatic side chain) replaces glycine, in the second threonine (polar neutral) replaces arginine (polar basic) and in the third isoleucine replaces sulphur containing methionine.

Constraints on DQαβ chain heterodimer formation mean that the great majority of DQw1 serogoup (DQ5 or 6) DQ molecules comprise a *DQA1*01* α-chain paired with either a *DQB1*05* or *DQB1*06* β-chain (Begovich, Klitz *et al.* 2000). This is the case for all the DQ5 and DQ6 cell lines used in this study. Conversely the DQw3 serogroup (DQ7 or 8) cell lines used in this study all express either *DQA1*03* or *DQA1*05* paired with a *DQB1*03* β-chain. Our observation of poor SPEA binding by *HLA-DQA1*03/*05* cell lines as compared to *HLA-DQA1*01* cell lines, irrespective of level of DQ expression, is likely therefore to be due to DQ α-chain polymorphism at sites of SPEA binding.

Table 3.1. Sequence alignment of the bacterial superantigen binding sites on the HLA-DQα chain (numbering corresponds to the *DQA1*0101* product) (Sundberg and Jardetzky 1999; Marsh, Parham *et al.* 2000).

	Superantigen binding sites on the DQ alpha chain																
DQA1	**16**	**20**	**21**	**22**	**23**	**39**	**40**	**41**	**42**	**58**	**60**	**61**	**63**	**64**	**66**	**67**	**70**
0101/2	Y	G	P	S	G	L	E	R	K	D	Q	G	L	R	M	A	K
0103								K									
0301/2												F		T	I		
0501/5							G					F		T	I		

A similar analysis of HLA-DR β-chain polymorphism at sites of predicted SEA binding reveals a molecular basis for our observation of differences in SEA binding by DR4, 15 and 11. SEA binds the DR molecule via both the low affinity α-chain site and the high affinity β-chain site. Since the DR α-chain is non-polymorphic, differences in binding are likely to result from β-chain polymorphism.

The DR β-chain superantigen binding sites identified from crystallography of SPEC and SEH in complex with HLA-DR1 are virtually identical and are believed to be applicable to SEA and other superantigens which utilise the high affinity β-chain binding site (Li, Li *et al.* 2001; Petersson, Hakansson *et al.* 2001). Zinc stabilises the complex by cross linking SEH and the highly conserved βH81 on the β-chain of HLA-DR but other amino acids are involved. Table 3.2 shows the amino acid sequence of the *DRB1*0401* α-chain at sites implicated in superantigen binding and the substitutions which distinguish *DRB1*0402*, *DRB1*11* and *DRB1*15*. Only one

site directly implicated in SEA binding is polymorphic; aspartate (polar acidic) replacing glutamine (polar neutral) at β70. The work presented here therefore indicates that just as DQ α-chain polymorphisms determine SPEA binding to HLA-DQ, DR β-chain polymorphisms determine SEA binding to DR. Furthermore, even the most subtle allelic polymorphisms such as exist between *DRB1*0401* and *B1*0402* can result in differences in superantigen binding.

SEA, in common with other bacterial superantigens which use the zinc dependent β-chain binding site on HLA class II, and in common with TSST-1, also makes contact with antigenic peptide within the HLA class II peptide binding groove (Kim, Urban *et al.* 1994; von Bonin, Ehrlich *et al.* 1995; Hogan, VanBeek *et al.* 2001; Li, Li *et al.* 2001; Petersson, Hakansson *et al.* 2001). It is possible therefore that the differences described here in SEA binding to HLA-DR relate not to polymorphisms in the DR β-chain, but to differences in the bound antigenic peptide. Such an explanation would however not be applicable to the differences observed in SPEA binding to HLA-DQ since in the crystal structures of SSA and SEB, which model the class II α-chain binding site used by SPEA, no involvement of peptide has been identified (Jardetzky, Brown *et al.* 1994; Dessen, Lawrence *et al.* 1997; Sundberg and Jardetzky 1999).

Table 3.2. Sequence alignment of the bacterial superantigen binding sites on the HLA-DRβ chain (numbering corresponds to the *DRB1*0401* product) (Li, Li *et al.* 2001; Petersson, Hakansson *et al.* 2001)

Superantigen binding sites on the DR beta chain							
DRB1*	**69**	**70**	**76**	**77**	**80**	**81**	**84**
0401	E	Q	D	T	R	H	G
0402		D					
11		D					
15		Q					

3.5 Conclusions

The work described in this chapter demonstrates for the first time that allelic HLA class II polymorphisms determine the binding of bacterial superantigens. Since the effect of polymorphisms on binding was observed for both the low affinity α-chain binding superantigen SPEA and the high affinity β-chain binding superantigen SEA it is likely that class II polymorphisms influence the binding of all bacterial superantigens, with the possible exception of those which bind predominantly to the non-polymorphic DR β-chain, namely SEB and TSST-1. The functional implications of these differences in binding, and their biological significance, will be discussed subsequently in this thesis.

CHAPTER 4

Binding of bacterial superantigens to HLA class II in solution

4.1 Introduction

Analysis of the interaction between superantigens and the class II molecule in a cell free system has several important advantages over assays using cell surface expressed class II. Cultured cell lines express, and do so to a variable extent, other cell surface determinants which may bind superantigens, confounding the relationship between observed superantigen binding and class II mediated binding. HLA class I for example has been demonstrated to bind superantigen (Haffner, Zepter *et al.* 1996; Wright and Chapes 1999). Superantigen activation of T cells occurs in the presence of class II negative antigen presenting cells expressing the adhesion molecules ICAM-1 and LFA-3 (Lamphear, Stevens *et al.* 1998). In cellular studies of superantigen - class II binding, difficulties quantifying cell surface expression of class II may be compounded by cellular responses to superantigen ligation of class II, such as internalisation, upregulation and cell division (Chatila and Geha 1993).

There are precedents for assessing superantigen - class II interactions in isolation of other cell surface factors. Soluble mouse class II prepared using a baculovirus system and surface plasmon resonance has been used to assess the kinetics of superantigen binding (Kozono, Parker *et al.* 1995) and the class II α and β-chain binding sites of

SEA were defined by binding of mutant toxins to class II in solution (Tiedemann, Urban *et al.* 1995).

One setting in which the simplicity of a cell free assay of class II - superantigen binding may have great advantages is in the study of potential therapeutic agents which might disrupt this interaction. A number of novel therapeutic approaches to the treatment of superantigen disease have recently been described (Hong-Geller and Gupta 2003). Amongst these, peptides designed around the PROSITE PS00278 and PS00279 sequences described in chapter 1 (Sigrist, Cerutti *et al.* 2002) have been shown to block T cell proliferation in response to superantigens and to be protective in animal models (Arad, Levy *et al.* 2000; Visvanathan, Charles *et al.* 2001). The mechanism of action of such peptides is unclear. Binding to purified HLA-DR has been demonstrated for one peptide (Visvanathan, Charles *et al.* 2001) but the impact of this on superantigen binding has not been assessed.

The work presented in this chapter describes the development and use of a robust, simple ELISA based assay of superantigen – HLA class II binding. The results confirm the influence of HLA class II polymorphisms on the binding of SPEA to HLA-DQ and SEA to HLA-DR described in chapter 3. The assay is then used to investigate the ability of two putative superantigen antagonist peptides to block superantigen - class II binding. The first, the most potent peptide antagonist based on the PROSITE 279 sequence (Visvanathan, Charles *et al.* 2001), and the second a peptide fragment of the DQ alpha chain centred on the sequence identified in chapter 3 as being of central importance to HLA-DQ binding of SPEA.

4.2 Methods

4.2.1 Purification of HLA class II from B-LCLs

HLA class I, HLA-DR and HLA-DQ were affinity purified from cultured B-LCLs. Two purifications were performed, of HLA-DR11 and DQ7 from IDF cells and HLA-DR15 and DQ6 from PGF cells. In each case cells were cultured in a 5 litre stir flask, using complete RPMI under 5% CO_2 at 37°C. 2.5L of medium was harvested every 5 days, cells spun down to a pellet and frozen until after around three weeks a total yield of ~40g (~$2x10^{10}$) cells was accumulated. Cells were homogenised in ice cold Tris 10mM, DTT 1mM PMSF 0.1mM, pH8 buffer and centrifuged. The supernatant containing cell membranes was kept, the pellet resuspended in buffer and centrifuged again. This process was repeated 5-8 times and supernatants pooled until the pellet was white indicating that the majority of cell membranes had been extracted into solution. Membrane bound proteins were pulled out of suspension by ultracentrifugation at 175,000g for 40min and resuspended in buffer. Membrane bound proteins were then detergent extracted by addition of nonidet-NP40 and cell membranes removed by further ultracentrifugation. The supernatant of solubilised cell membrane proteins thus obtained was then passed over a series of five affinity purification columns; non-specific CL-4B, non-specific protein A sepharose, anti class I (W6/32), anti HLA-DR (L243), anti HLA-DQ (SPVL-3). The columns were washed twice with 20mM MOPS, 140mM NaCl, 0.1% deoxycholic acid (DOC), four times with 10mM Tris, 0.1% DOC, pH 8, and eluted with 50mM glycine, 0.1% DOC pH 11.5. Column eluates were neutralised with 2M glycine pH 2.0 and dialysed

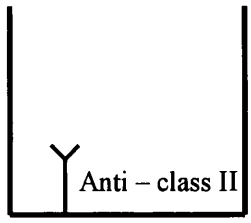
against 10mM Tris, 0.1% DOC, pH8.0 for 24 hours and then concentrated using an Amicon stir cell PM30 membrane.

4.2.2 Superantigen – soluble HLA class II binding assay (Fig. 4.1)

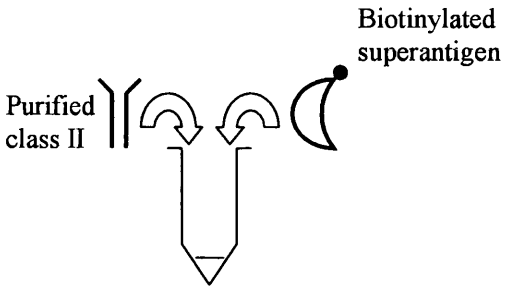
Wells of a 96 well C-bottomed Maxisorp ELISA plate (Nunc, UK) were coated with 100µl of L243 (anti-HLA-DR) or SPV-L3 (anti-HLA-DQ) by overnight incubation at 4°C. After washing in PBS 0.1% Tween-20, wells were blocked using 400µl/well of PBS 1% BSA for 1 hour at room temperature. A series of reaction tubes was set up containing 20µl of 30µg/ml purified HLA class II and biotinylated superantigen (SPEA, SEA or SEB) at a range of concentrations (0-100µg/ml) and left to incubate overnight at 4°C. Negative control reaction tubes were set up containing biotinylated superantigen alone at the same concentrations. After overnight incubation, the volume in each reaction tube was made up to 100µl with PBS 1% BSA and duplicates of 50µl/well were set up in the ELISA plate. After 1 hour incubation at room temperature wells were washed 6 times in PBS 0.1% Tween and 50µl/well of avidin-HRP (Jackson, PA) 1:2500 in PBS 1% BSA was added. After a further 1 hour incubation at room temperature wells were washed 6 times and 50µl/well tetramethyl benzidine (TMB) (Sigma, UK) was added. Colour change was stopped after 5 minutes using 50µl (1M) H_2SO_4 and read on a Labsystems Multiscan plate reader at 450nM.

Fig. 4.1. ELISA of superantigen HLA class II binding

1. ELISA plate coated with HLA class II antibody



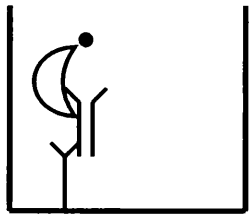
2. Varying concentrations of biotinylated superantigen allowed to bind purified HLA class II in solution



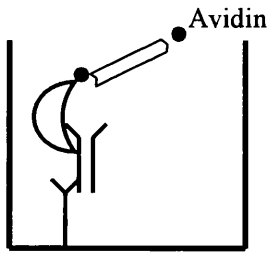
Test = SAg + class II

Control = SAg + PBS

3. HLA class II - superantigen complexes captured



4. Captured complexes detected using avidin - HRP



4.2.3 Superantigen antagonist peptides

Peptide 6343 (Visvanathan, Charles *et al.* 2001), a 12-mer peptide based on the PROSITE PS00279 sequence and a scrambled 12-mer control were the gift of Dr. Kumar Visvanathan, Murdoch Childrens' Research Institute, PO Box 1100, Parkville, Victoria, 3052, Australia.

20-mer peptides representing the DQA1*01(53-73) sequence and a three amino acid mutant containing the three substitutions differentiating DQA1*01 from DQA1*05 within this sequence were synthesised by Biosyn Inc. TX. Peptides were prepared as 15mg/ml stock solutions in PBS, aliquoted and frozen at -20°C until use. Peptide sequences and molecular weights are shown in Table 4.1

Table 4.1. Superantigen antagonist peptides

*DQA1*01*(53-73)	K F G G F D P Q G A L R N M A V A K H N L	2.24kD
*DQA1*01*(53-73) mut.	K F G G F D P Q F A L T N I A V A K H N L	2.26kD
6343	C M Y G G V T E H E G N	1.1kD
Scrambled peptide	E C E G N H M G G Y V T	1.1kD

4.3 Results

4.3.1 Characterisation of purified HLA class II

The protein concentration of affinity purified HLA class II was measured using the BCA-200 kit, (Pierce, IL). PAGE of column eluates under reducing and denaturing conditions confirmed the presence of two bands either side of the 30kD marker corresponding to the molecular weights of the class II alpha and beta chains (Fig. 4.2a). The class II alpha chain is heavily glycosylated and as a result the heavier alpha chain band is more diffuse than the lighter beta chain. PAGE under non-denaturing, non-reducing conditions (no DTT in the sample buffer and sample not boiled prior to gel loading) demonstrated that most of the protein is present as alpha-beta chain heterodimers of molecular weight ~60kD under these conditions (Fig 4.2b). Western blot analysis using antibodies to HLA class II alpha and beta chains was used to confirm the identity of the bands each side of the 30kD marker (Fig 4.3).

4.3.2 Characterisation of superantigen-HLA class II binding assay

Figure 4.4a shows a typical readout of SEB-DR11 binding, illustrating the relationship between superantigen concentration and optical density. The specificity of the interaction was further confirmed by the observation that binding of biotinylated SEB could be blocked using excess unconjugated SEB (Fig 4.4b).

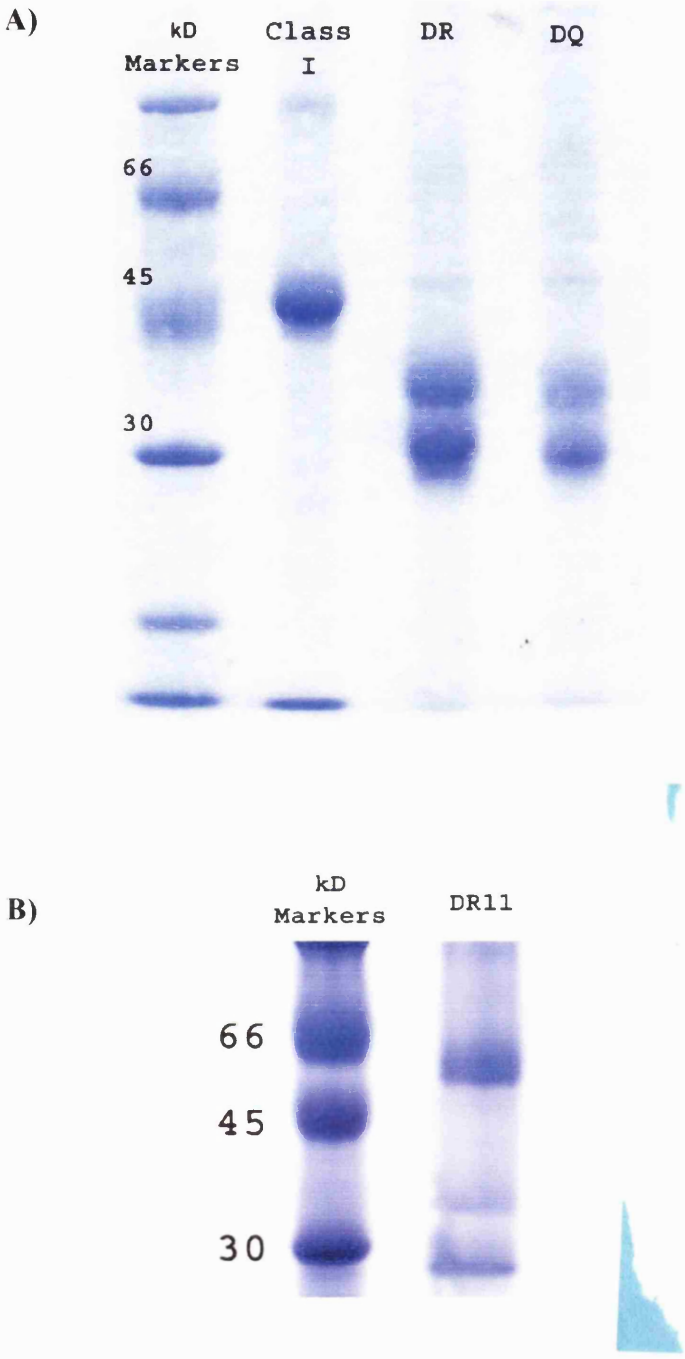


Fig. 4.2. SDS-PAGE and native gel of purified HLA class II. A) Class I, HLA-DR11 and DQ3 purified from IDF cells. **B)** non reduced, non denatured gel showing DR11 running predominantly as heterodimer under these conditions.

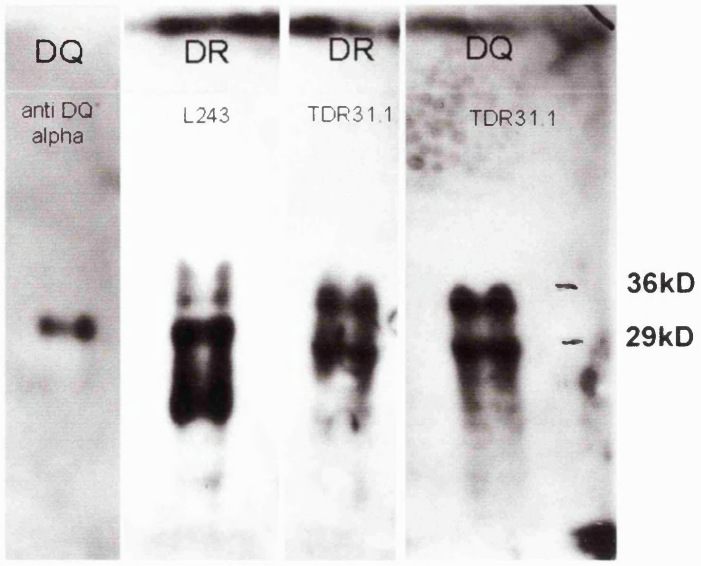


Fig. 4.3. Western blot of purified HLA-DQ6 and HLA-DR15. The antibody TDR 31.1 binds the class II β-chain to produce a band just below the 29kD marker. L243 (anti DR α-chain) and the anti DQ α-chain antibody produce a band around 33kD corresponding to the weight of the class II α-chain. Non-specific bands are seen at around 35kD in the L243 and TDR 31.1 lanes and around 18kD in the L243 lane.

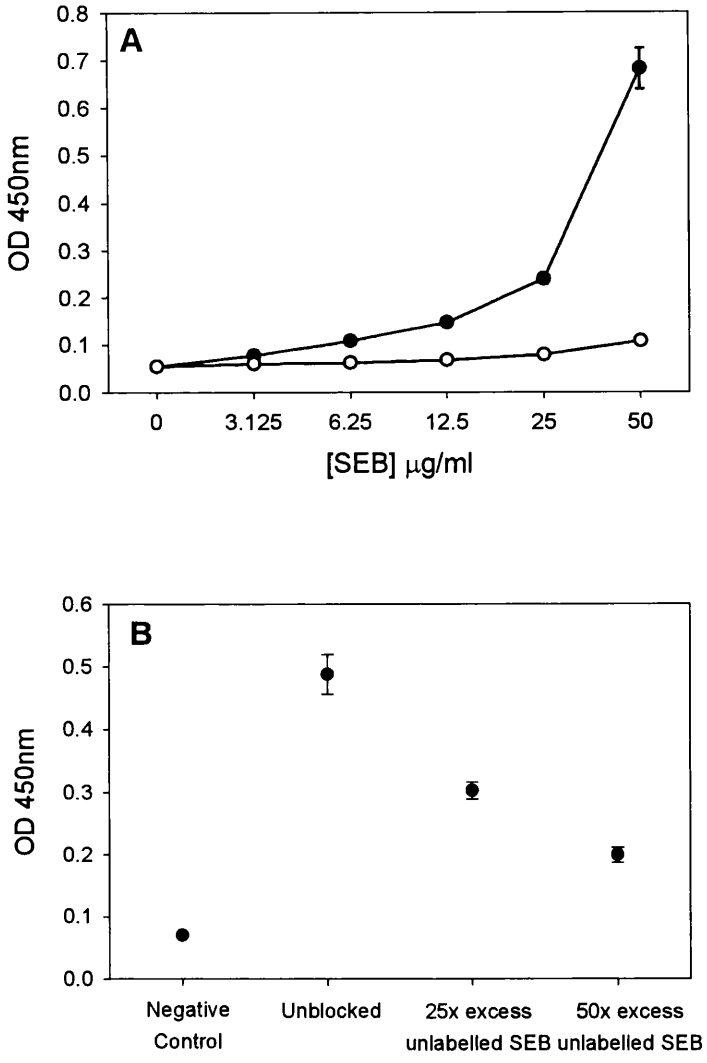


Fig. 4.4. Binding of biotinylated SEB to HLA-DR11 in solution. **A)** relationship between SEB concentration and optical density (●) control (capture of SEB in the absence of class II) (○). **B)** influence of excess unconjugated SEB on binding of 25µg/ml conjugated toxin. Points show mean +/- 1SD.

The binding of SEA to HLA-DR is mediated through a zinc-dependent binding site on the DR β-chain (Schad, Zaitseva *et al.* 1995; Li, Li *et al.* 2001; Petersson, Hakansson *et al.* 2001) while SEB binding is through a zinc-independent binding site on the DR α-chain (Jardetzky, Brown *et al.* 1994). As an additional control to confirm the specificity of the assay we therefore determined the effect of zinc chelation, using 1mM EDTA, on binding of SEB and SEA to purified HLA-DR. As predicted, 1mM EDTA completely abrogated the binding of SEA to HLA-DR11 but had no effect on HLA-DR binding of SEB Figure 4.5 shows a representative experiment.

4.3.3 Influence of HLA-DR polymorphism on SEA and SEB binding

Comparison was made of SEA binding to different DR molecules. HLA-DR11 and 15 were purified as described above. HLA-DR4 purified as described above from PRIESS cells was donated by Dr Mark Peakman, Department of Immunology, Kings, Guys and St. Thomas' School of Medicine, London. In repeated comparisons of SEA binding to these DR molecules, differential SEA binding was observed with DR15 and 4 showing comparable, higher binding of SEA than DR11. In the representative experiment shown in figure 4.6 an approximately five-fold higher concentration of SEA was needed in the presence of DR11 to give a level of binding comparable to that observed in the presence of DR4 (Fig. 4.6a). In contrast, no distinct differences in the binding of SEB were apparent comparing these three DR molecules (Fig. 4.6b).

4.3.4 Influence of HLA-DQ polymorphism on SPEA binding

In order to confirm the observation of differential SPEA binding by HLA-DQ molecules, comparison was made of SPEA binding to purified HLA-DQ3 and DQ6. In three separate experiments assessing HLA-DQ binding of SPEA over a range of

concentrations from 1-50μg/ml, greater binding of SPEA by DQ6 than DQ3 was observed. In the representative experiment shown in figure 4.7 an approximately four-fold higher concentration of SPEA was needed in the presence of DQ3 to give a level of binding comparable to that observed in the presence of DQ6.

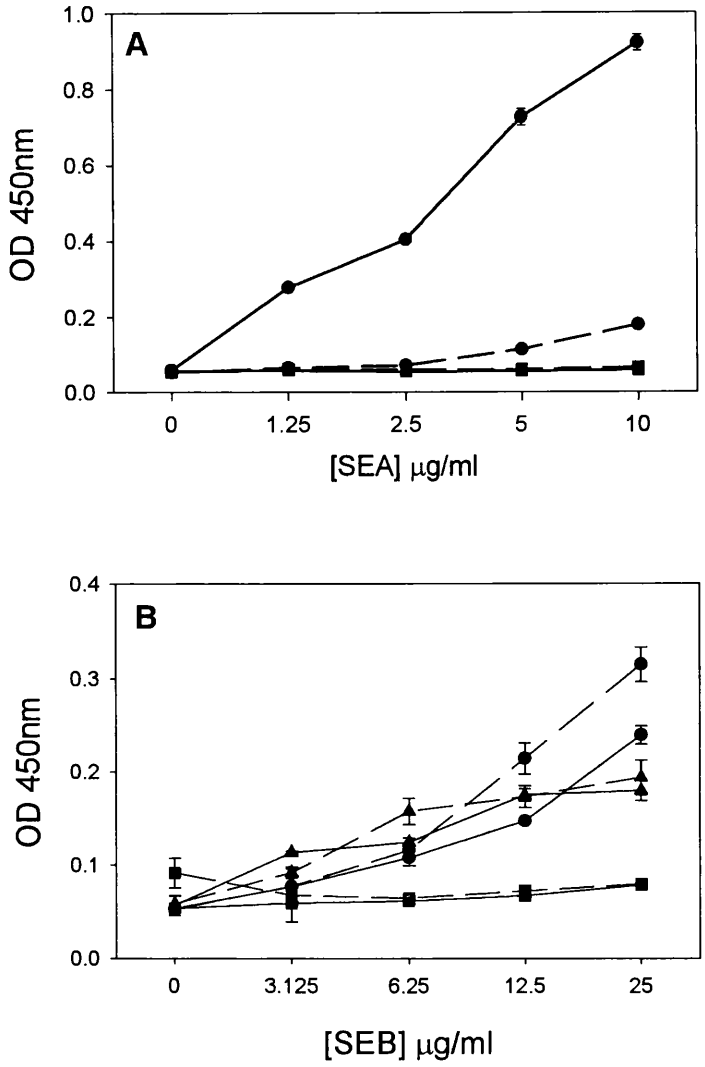


Fig. 4.5. Influence of zinc chelation on HLA-DR binding of superantigens. A) SEA – HLA-DR11 binding (●) in the presence (----) or absence (——) of 1mM EDTA, control (■). **B)** SEB – HLA-DR11 binding (●) and SEB – DR15 binding (▲) in the presence (----) or absence (——) of EDTA, control (■). Points show mean +/- 1SD.

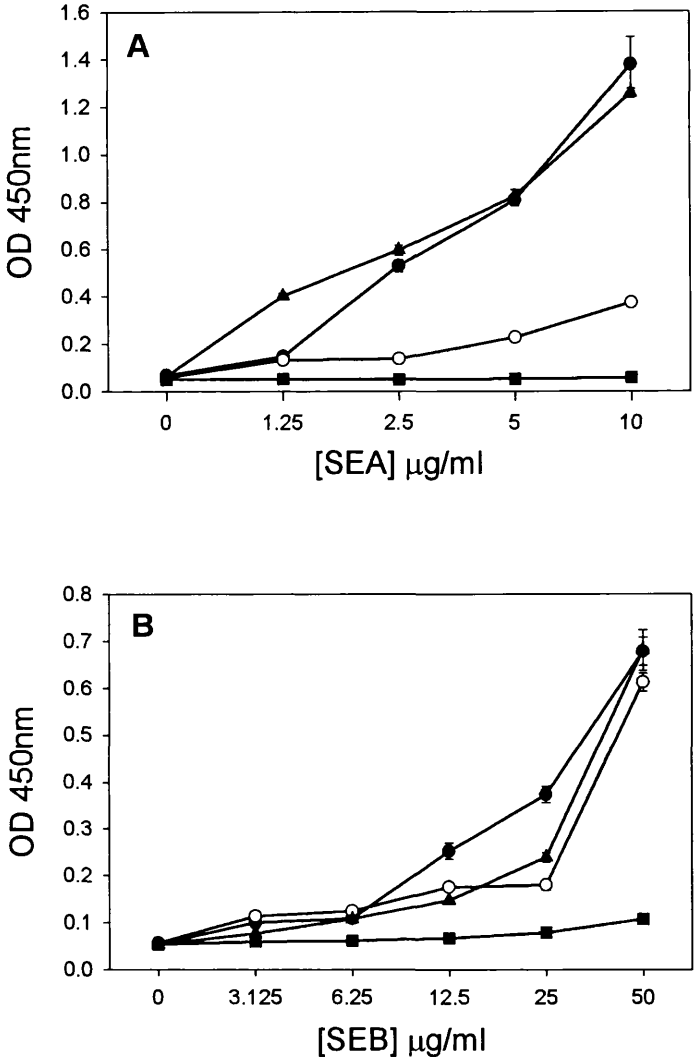


Fig. 4.6. SEA and SEB binding by different HLA-DR molecules. **A)** SEA and **B)** SEB, HLA-DR 11 (●), HLA-DR15 (▲), HLA-DR4 (○), control (■). Points show mean +/- 1SD.

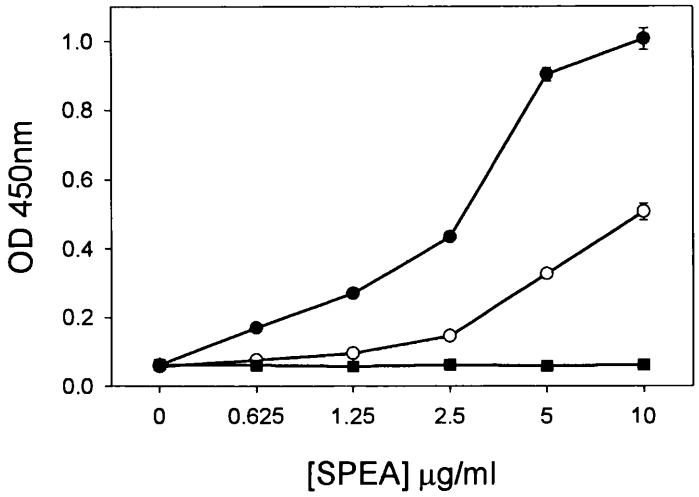


Fig. 4.7. SPEA binding by different HLA-DQ molecules. SPEA binding to HLA-DQ6 (●), DQ3 (○) and control (■). Points show mean +/- 1SD.

4.3.5 Influence of zinc chelation on HLA-DQ binding of SPEA

HLA-DQ binding of SPEA is known to occur at a site on the DQ α-chain. By analogy with the SEB interaction with the DR α-chain SPEA binding to HLA-DQ is thought to be zinc-independent. However, a zinc binding site has been identified in the crystal structure of SPEA (Papageorgiou, Collins *et al.* 1999; Earhart, Vath *et al.* 2000; Baker, Gutman *et al.* 2001). The functional importance of zinc for SPEA binding to HLA-DQ is unclear. To establish whether zinc might be important in this interaction we assessed binding in the presence or absence of 1mM EDTA. No impact of EDTA on SPEA binding of either HLA-DQ6 or DQ3 was detected (Fig. 4.8).

4.3.6 DQ alpha chain peptides

In order to conclusively test the hypothesis that HLA-DQ α-chain polymorphisms are associated with differences in SPEA binding, two 20 amino acid peptides were designed; one representing the *HLA-DQA1*01* α-chain 53-73; the other a three amino acid substitution mutant where the three amino acid differences distinguishing the *DQA1*01* α-chain and the *DQA1*05* α-chain had been made (Table 4.1). Using different concentrations of peptide to coat wells of a 96 well ELISA plate it was possible to demonstrate concentration dependent capture of biotinylated SPEA by both the DQA1*01(53-73) and the three substitution mutant compared with BSA blocked wells. Furthermore binding to the mutant peptide was markedly lower than to the *DQA1*01*(53-73) peptide. This observation was consistent in three separate experiments. In the representative experiment, a two log higher concentration of the mutant peptide was required to achieve capture of SPEA comparable with the unmutated peptide (Fig. 4.9).

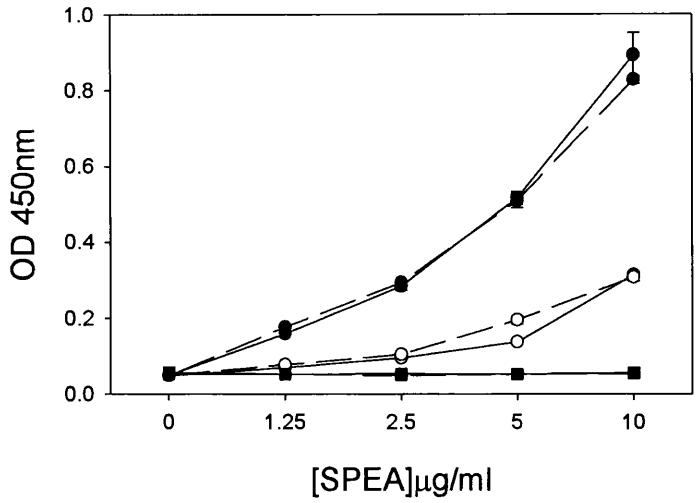


Fig. 4.8. Influence of zinc chelation on HLA-DQ binding of SPEA. DQ6 (●), DQ3 (○) or control (■) in the presence (----) or absence (——) of EDTA. Points show mean +/- 1SD.

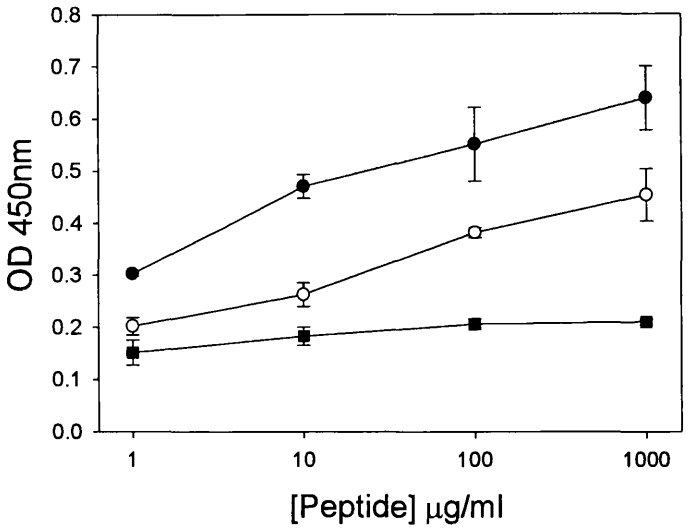


Fig. 4.9. Binding of SPEA to HLA-DQ alpha chain peptides. HLA-DQA1*01 (53-73) (●), HLA-DQA1*01(53-73) mutant (○) or control (■).Points show mean +/- 1SD.

4.3.7 Peptide inhibitors of superantigen- class II binding

Three groups have recently published work which indicates that peptides based on the two highly conserved PROSITE superantigen sequences have therapeutic potential as superantigen antagonists. Such peptides appear to be protective in animal models of superantigen toxicity (Arad, Levy *et al.* 2000; Visvanathan, Charles *et al.* 2001; Shupp, Jett *et al.* 2002) but their mechanism of action is unclear. One group has proposed that they inhibit co-stimulatory pathways involved in superantigen induced T cell activation (Arad, Levy *et al.* 2000). A second group has suggested that their mechanism of action could be through inhibition of superantigen membrane transcytosis (Shupp, Jett *et al.* 2002). A third group have suggested that these peptides may inhibit superantigen binding of MHC class II (Visvanathan, Charles *et al.* 2001). In order to determine whether such peptides do block binding of superantigens to HLA class II we performed assays of superantigen - class II binding in the presence of peptide 6343, using as controls, either scrambled peptide or an alanine trimer (Sigma Aldrych, UK). Additionally, in view of the ability of the peptide *DQA1*01*(53-73) to bind SPEA, the possibility that this peptide might act as a competitive antagonist to HLA class II in superantigen binding was also investigated.

The binding of SPEA to purified DQ6 was found to be inhibited by peptide 6343 by 50% although inhibition was not detectable at concentrations below 313μg/ml and was maximal at 2500μg/ml (Fig 4.10a). The alanine trimer control produced no inhibition. The *DQA1*01*(53-73) peptide also produced around 50% inhibition of SPEA binding at 5000μg/ml with blocking detectable above 625μg/ml. This

specificity of this inhibition is apparent from the much lower degree of blocking seen with the *DQA1*01*(53-73) mutant peptide which was only 10% at 5000μg/ml (Fig 4.l0b).

The binding of SEA to purified HLA-DR15 was, as expected, not inhibited by either the *DQA1*01*(53-73) peptide or its mutant control. SEA-DR15 binding was inhibited by peptide 6343 by about 60%, however scrambled peptide control was also found to block binding to a significant extent; approximately 30% at a concentration of 2500μg/ml (Fig 4.11).

To confirm that blocking of class II superantigen binding by the peptides and controls correlated with inhibition of superantigen induced PBMC proliferation, PBMCs were stimulated with SPEA in the presence or absence of the peptides and their controls at a range of concentrations (Fig 4.12). Modest, dose dependent, inhibition of SPEA proliferation was observed with *DQA1*01*(53-73) peptide which was not observed with the mutant peptide control (Fig 4.12a). A more marked, dose dependent, inhibition of SPEA proliferation was observed with peptide 6343 but in keeping with the scrambled peptide's property of blocking SPEA - DQ binding, the scrambled peptide also showed a trend towards dose dependent blocking of SPEA proliferation (Fig 4 .12b).

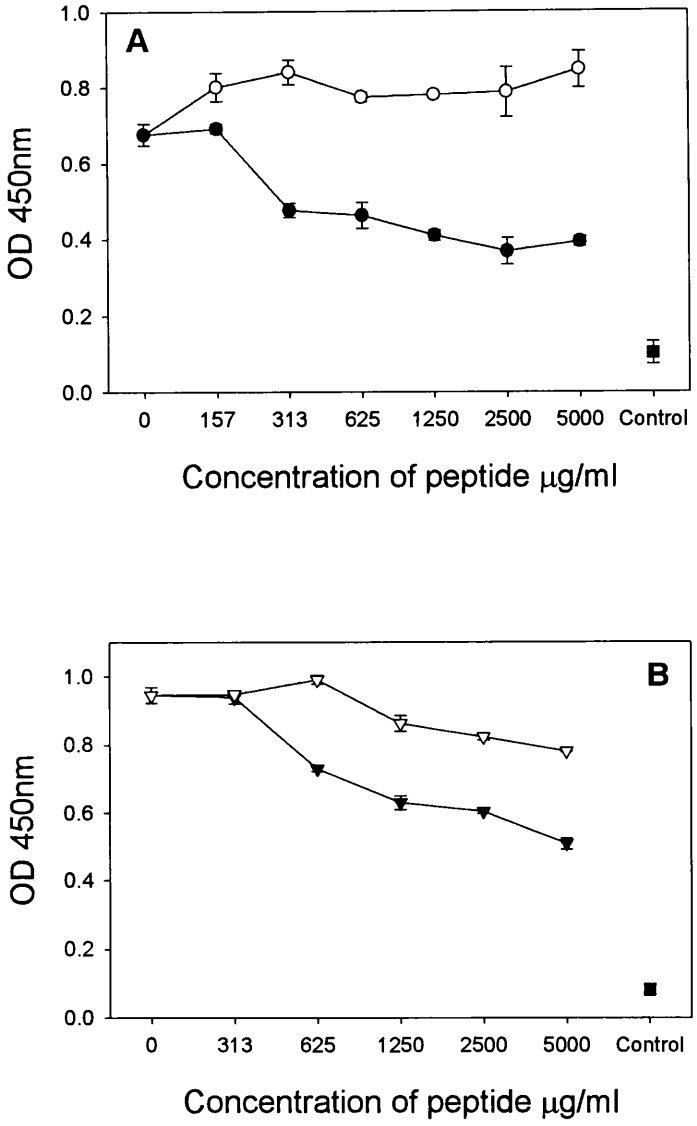


Fig. 4.10. Inhibition of SPEA binding to HLA-DQ6 by superantigen antagonist peptides. The effect on binding of SPEA (5µg/ml) to HLA-DQ6 (20µg/ml) of peptides is shown. **A)** Peptide 6343 (●) and alanine trimer control (○), negative control (second layer only) (■). **B)** DQA1*01(53-73) (▼) and mutDQA1*01(53-73) (▽), negative control (second layer only) (■).Points show mean +/- 1SD.

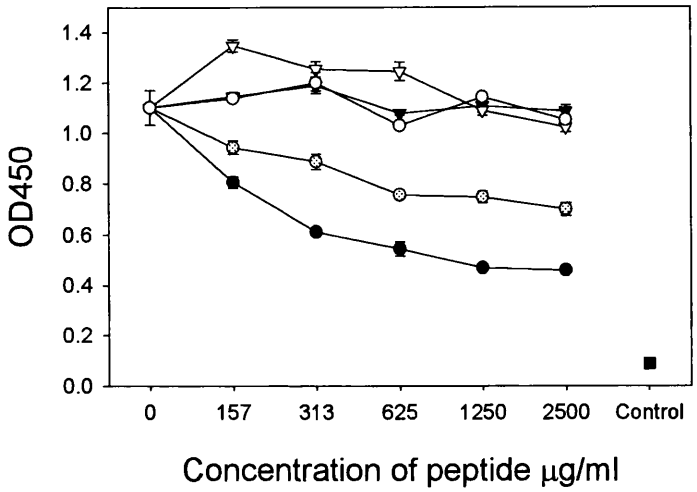
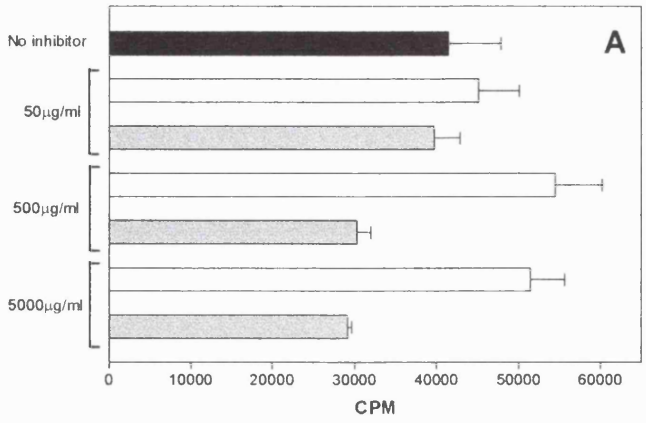


Fig. 4.11. Inhibition of SEA binding to HLA-DR15 by superantigen antagonist peptides. The effect on binding of SEA (5µg/ml) to purified DR15 (20µg/ml) of superantigen antagonist peptide 6343 (●), scrambled peptide (●), alanine trimer (○), DQA1*01(53-73) (▼), or mutDQA1*01(53-73) (▽) is shown. Points show mean +/- 1SD.



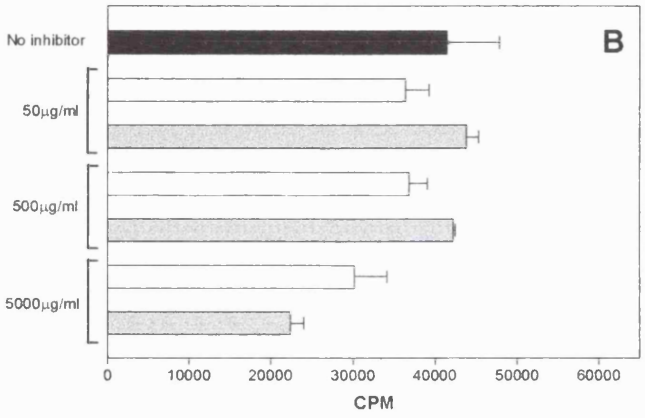


Fig. 4.12. Inhibition of PBMC response to SPEA by superantigen antagonist peptides. PBMCs stimulated with SPEA (100ng/ml) in the presence of various concentrations of inhibitor or control. **A)** no inhibitor (■), DQA1*01(53-73) (▒) or mutated peptide (□), **B)** no inhibitor (■) ,6343 (▒) or scrambled peptide (□). Bars show mean +/- 1SD.

4.4 Discussion

Although for many purposes it is desirable to study the interaction between MHC class II and superantigens with the class II molecule as physiologically normal as possible, there are several advantages of a cell free assay of superantigen - class II binding. In particular such an assay avoids the potential for other cell surface molecules such as class I and adhesion molecules to confound the observed superantigen binding.

The results presented in this chapter, which show differences in SPEA binding to DQ6 (*DQA1*0102/DQB1*0602*) and DQ7 (*DQA1*0301/DQB1*0301*) and SEA binding to DR15 (*DRA1*0102/DRB1*1501*) DR4 (*DRA1*0101/DRB1*0401*) and DR11 (*DRA1*0101 /DRB1*1103*), confirm the finding of allelic differences in HLA class II binding of these superantigens. The use of peptide fragments of the DQ α-chain covering the sites of SPEA binding has made it possible to focus precisely on the polymorphisms which distinguish *DQA1*01* from *DQA1*03* and *DQA1*05*. The finding that substituting F61G, T64R, 166M in the peptide *DQA1*01*(53-73) was associated with marked reduction of SPEA binding to this peptide confirms that the differences in SPEA binding to HLA-DQwl cell lines and DQw3 cell lines observed in chapter 3 is indeed due to these polymorphisms in the DQ α-chain and can not be attributed to polymorphisms elsewhere in the DQ molecule, differences in antigenic peptide or level of DQ expression.

The finding that zinc chelation, using EDTA, very substantially reduced the observed binding of SEA to HLA-DR is entirely in keeping with the known zinc dependence of the high affinity bond between SEA and the HLA class II α-chain. Many

superantigens only achieve full activity by cross-linking class II on the surface of APCs (Roussel, Anderson *et al.* 1997; Wright and Chapes 1999). Although SPEA only interacts via the class II molecule via a low affinity zinc-independent mechanism, the recent observations that SPEA forms dimers in solution and contains a zinc binding pocket has raised the possibility that zinc dependent dimerisation might play a role in the class II binding of this superantigen (Papageorgiou, Collins *et al.* 1999; Earhart, Vath *et al.* 2000; Baker, Gutman *et al.* 2001).

The central importance of the class II interaction with superantigen makes it an attractive target for novel therapies directed against superantigen mediated disease (Hong-Geller and Gupta 2003). To have therapeutic potential, both peptide and antibody inhibitors of superantigen presentation would need not to disrupt the presentation of conventional antigen. The peptide inhibitors of superantigens based on the PROSITE PS00278 and PS00279 sequences have been shown to block T cell proliferation in response to superantigens (Arad, Levy *et al.* 2000; Visvanathan, Charles *et al.* 2001). The most potent peptide inhibitor of proliferation reported by Visvanathan *et al* was peptide 6343 (CMYGGTEHEGN), based on PS00279. This peptide showed up to 80% inhibition of T cell proliferation in response to all superantigens against which it was tested including TSST-1, a toxin in which the PS00279 sequence is not found. The peptide also protected d-galactosamine sensitised mice from lethal challenge with SEB when given prior to challenge. Using purified DR1 Visvanathan *et al* showed binding to 6343 to class II but did not demonstrate inhibition of superantigen binding. (Visvanathan, Charles *et al.* 2001) The results presented here confirm that the peptide 6343 does indeed block the

binding of both SPEA to HLA-DQ and SEA to HLA-DR. The concentration required to achieve this effect was high at between 3 $x10^{-4}$ and $4.5x10^{-3}$M. However these concentrations are comparable with those required to inhibit PBMC proliferation in both the Visvanathan *et al* published study of this peptide and our own observations. In terms of molar excess this represents 1000 to 15000 fold excess compared to the class II concentration used ($3x10^{-7}$M). Moreover at this concentration range the scrambled peptide was also observed to cause less but still appreciable inhibition of class II superantigen binding. Accordingly we found that at this concentration range the scrambled peptide caused some inhibition of SPEA induced PBMC proliferation. The DQA1*01(53-73) peptide was observed to cause inhibition of SPEA binding of HLA-DQ over a similar range of concentrations from $2.6x10^{-4}$ to $2.2x10^{-3}$M. This effect was specific in two regards. Firstly, it was confined to blocking of SPEA binding to HLA-DQ and was not apparent when looking at SEA binding to HLA-DR. Secondly, the mutated peptide which showed much reduced SPEA binding also showed much less inhibition of the SPEA-DQ interaction. In keeping with these observations the peptide DQA1*01(53-73) blocked SPEA induced PBMC proliferation in a dose dependent manner and the mutant peptide control did not.

4.5 Conclusions

The work presented in this chapter defines an ELISA based assay of superantigen binding to HLA class II using affinity purified, peptide loaded, conformationally intact HLA-DR and DQ. Using the assay to compare superantigen binding to different HLA-DR and DQ molecules the findings of chapter 3 regarding differential binding of SPEA by different DQ alleles and SEA by different DR alleles have been confirmed. Using a peptide based around the DQ alpha chain at sites of superantigen binding and a second peptide differing from the first by three amino acid substitutions, the importance of DQ α-chain polymorphisms in determining the binding of SPEA has been confirmed. The assay described has potential as a tool to study novel therapeutic agents which disrupt superantigen - class II binding. This has been demonstrated using two superantigen antagonist peptides which may act in this way.

CHAPTER 5

Influence of HLA class II polymorphisms on T cell response to superantigens

5.1 Introduction

The importance of the differences in HLA class II – superantigen binding identified in chapters 3 and 4 arises from any associated differences which may exist in the response to superantigens. Intuitively, it seems likely that differences in class II affinity for a superantigen will be associated with differences in the magnitude of the T cell response. There are a number of lines of evidence which suggest that this is likely to be the case. Firstly, the bacterial superantigens implicated in causing human disease all have higher affinities for HLA class II than mouse MHC class II (Herman, Croteau *et al.* 1990) and splenocytes from mice transgenic for HLA class II show markedly increased responsiveness to bacterial superantigens (Yeung, Penninger *et al.* 1996; Sriskandan, Unnikrishnan *et al.* 2001). Secondly, superantigens vary greatly in their potency, with half maximum stimulation values for human PBMCs (P_{50}(h)) varying from 0.02pg/ml for SMEZ to 50pg/ml for SPEH (Proft and Fraser 2003). Differences in superantigen - class II affinity are likely to be responsible for much of this variation. There is a general correlation between superantigen potency and affinity both for class II and TCR (Mollick, Chintagumpala *et al.* 1991; Li, Llera *et al.* 1998). While the overall stability of the trimolecular complex formed by class II,

superantigen and TCR may ultimately determine T cell response (Seth, Stern *et al.* 1994), it has been suggested for example, that the greater potency of SEB than SEC may be explained by the high affinity of SEB for HLA-DR more than compensating for the relatively low affinity of the SEB-TCR interaction (Leder, Llera *et al.* 1998). Thirdly, studies using mutant and naturally occurring variants of superantigens have noted that mutations impacting on class II binding affinity are reflected in changes in superantigen potency (Kline and Collins 1996; Leder, Llera *et al.* 1998).

While the Vβ repertoire changes associated with an individual superantigen are generally regarded as a fixed property, the class II involved in superantigen presentation may have an effect here also. For example, Surman *et al* reported differential responses in murine Vβ14 but not Vβ8.2 T cell hybridomas to SEB presented by H2-Ed or H2-Ek (Surman, Deckhut *et al.* 1994). Wen *et al* demonstrated that the effect of class II on Vβ response to a superantigen was dependent on the superantigen-TCRVβ affinity such that low affinity interactions were influenced by class II polymorphisms and high affinity interactions were not (Wen, Blackman *et al.* 1995). Finally, Newton *et al* demonstrated that superantigen mutations targeted at sites of class II binding could differentially effect the Vβ specificity of the T cell response (Newton, Dohlsten *et al.* 1996).

The work described in this chapter addresses the question of whether the differences in class II binding of the superantigens SPEA and SEA, described in the preceding chapters, are associated with differences in the T cell response to these superantigens. Using three distinct approaches, the greater binding of SPEA by *HLA-DQA1*01* than *DQA1*03/05* is shown to be associated with more marked T cell proliferation and

cytokine production. Using one of these approaches, differences in the magnitude of T cell response to SEA are demonstrated for DR4 and DR11. Studying the differential effect of HLA class II on the Vβ specific response to SPEA and SEA, a previously unreported relationship is observed between superantigen concentration and the Vβ specific T cell response. Differences in the relative expansion of different Vβs by both SPEA and SEA are shown to result from class II differences such that the Vβ response to a low concentration of superantigen in the presence of a high binding class II resembles that seen at approximately log-fold higher concentrations when presentation is by a low binding class II molecule.

5.2 Methods

5.2.1 DO11.10 mice

DO11.10 mice are transgenic for a TCR that recognises a chicken ovalbumin-derived peptide $OVA_{(323-329)}$ presented by H2-Ad. This TCR is encoded by transgenes encoding mVβ8.2/Vα13.1 (Murphy, Heimberger *et al.* 1990). Since mVβ8.2 is the primary mVβ targeted in the murine response to SPEA (Imanishi, Igarashi *et al.* 1990), splenocytes from DO11.10 mice may be exploited as a uniform population of responder cells in SPEA proliferation assays. In studying the influence of HLA class II polymorphism on the T cell response to SPEA, use of DO11.10 splenocytes takes advantage of the poor binding and presentation of SPEA by murine MHC class II (Herman, Croteau *et al.* 1990; Muller-Alouf, Alouf *et al.* 1992).

Spleens were dissected out of freshly culled mice and immersed in medium (complete RPMI containing 5μM β-mercaptoethanol). Splenocytes were then flushed out from each spleen by repeatedly injecting medium through the spleen tissues until blanched. Each spleen was then discarded, splenocytes pooled from two or three mice depending on the number of cells required, counted and plated out in flat bottomed 96 well plates for proliferation assay as described in chapter 2.

5.2.2 T cell purification

T cells were purified from human donor PBMCs by Dynal™ bead (Dynal Biotech, Oslo, Norway) negative selection of B cells and other class II positive cells (Lavoie, McGrath *et al.* 2001). $5x10^6$ PBMCs were spun to a pellet and resuspended at a count of $5x10^7$ cells/ml in L243 (mouse anti HLA-DR), WR18 (mouse anti HLA-DP, DQ,

DR), Leu 14 (mouse anti human CD14) and Leu 19 (mouse anti human CD19). After 1 hour incubation at 4°C cells were washed twice and two rounds of depletion of antibody bound cells were performed using anti-mouse immunoglobulin Dynal™ beads according to the manufacturer's instructions (Dynal Biotech, Oslo, Norway). The purity of remaining T cells was assessed in two ways. Firstly, two colour FACS analysis for CD3 and HLA class II was performed on unmanipulated PBMCs and purified T cells. Secondly, in each experiment using purified T cells, it was confirmed that the purified T cells failed to proliferate in response to superantigen unless co-incubated with exogenous class II positive APCs.

5.2.3 Preparation of Antigen Presenting Cells

B-LCLs and HLA-DQ transfectant BLS lines were used as APCs to present superantigen to responder cells; either murine DO11.10 splenocytes or purified human T cells. APCs were either irradiated or fixed with paraformaldehyde prior to incubation with responder cells. Irradiation was by exposure to 1800 rads. APCs were recultured for 24 hours after irraditation, prior to use, to ensure cell division had ceased prior to counting and plating out with superantigen and responder cells.

Where superantigen presentation was to be by paraformaldehyde fixed APCs, cells were incubated with superantigen for 40min at 4°C, washed twice in sterile PBS and then fixed by incubation in 1% paraformaldehyde at room temperature for 5 minutes. After two further washes in complete tissue culture medium cells, were resuspended at the appropriate cell concentration and plated out with purified T cells in 96 well plates.

5.2.4 HLA typing of healthy donors

Thirty five healthy donors from the Departments of Infectious Diseases or Immunology at the Hammersmith hospital were venesected and samples sent to the tissue typing laboratory at the Hammersmith hospital for HLA typing. Eight donors were either HLA-DQ5,5, DQ5,6 or DQ6,6 and therefore assumed to be *HLA-DQA1*01* homozygous, six were either HLA-DQ7,7, DQ7,8 or DQ8,8 and therefore assumed to be *HLADQA1*03/05*. In experiments comparing the response to SPEA of PBMCs from *DQA1*01* homozygous donors with *DQA1*03/05* donors, PBMCs from three donors of each HLA type were used.

5.2.5 FACS analysis of T cell Vβ repertoire

Fresh PBMCs, purified T cells and superantigen or PHA stimulated cells were analysed for the proportion of the T cell repertoire made up by individual Vβ types. Monoclonal antibodies against TCR Vβ cojugated with, phycoerythrin (PE), or in some instances FITC were used (Serotec, Oxford, UK and Beckman Coulter, High Wycombe, UK). Cells were co-stained with anti CD3-FITC or anti CD4-FITC or -PE and/or anti CD8-FITC or - peridinin chlorophyll (PerCP) (all supplied by Pharmingen San Diego CA) and analysed by two or three colour flow cytometry as described in chapter 2.

5.3 Results

A) Influence of class II on the magnitude of the T cell response to superantigens

The ability of high and low SPEA-binding HLA-DQ molecules to support SPEA activation of T cells was assessed using three approaches.

5.3.1 Influence of HLA-DQ on DO11.10 splenocyte response to SPEA

The aim of this first approach was to control maximally for variability attributable to donor T cell differences. This was achieved by using HLA homozygous B-LCLs and HLA-DQ transfected BLS cell lines to present SPEA to murine TCR Vβ8.2 transgenic responder T cells, mVβ8.2 being one of the murine Vβ families targeted in the response to SPEA. B-LCLs expressing *DQA1*01* were found to support higher levels of splenocyte proliferation in response to SPEA than those expressing *DQA1*05*. A two log higher concentration of SPEA was required in the presence of *DQA1*05* APCs to produce proliferation comparable with *DQA1*01* despite comparable levels of HLA-DQ expression. Two representative experiments are shown in figure 5.1. Similarly BLS cells transfected to express *DQA1*01* supported much greater T cell proliferation than those transfected to express *DQA1*03*. 100ng/ml SPEA was required in the presence of *DQA1*03* transfectants to produce T cell proliferation comparable with 0.1ng/ml in the presence of *DQA1*01* transfectants (Fig. 5.2). Corresponding differences in TNFα, IFNγ and IL-4 production were also observed comparing *DQA1*01* and *DQA1*03/05* APCs. Figure 5.3 and 5.4 show results from experiments using B-LCLs and BLS transfectants respectively as APCs. Production of all three cytokines in response to SPEA was greater in the presence of *HLA-DQA1*01* compared with *DQA1*03/05* APCs.

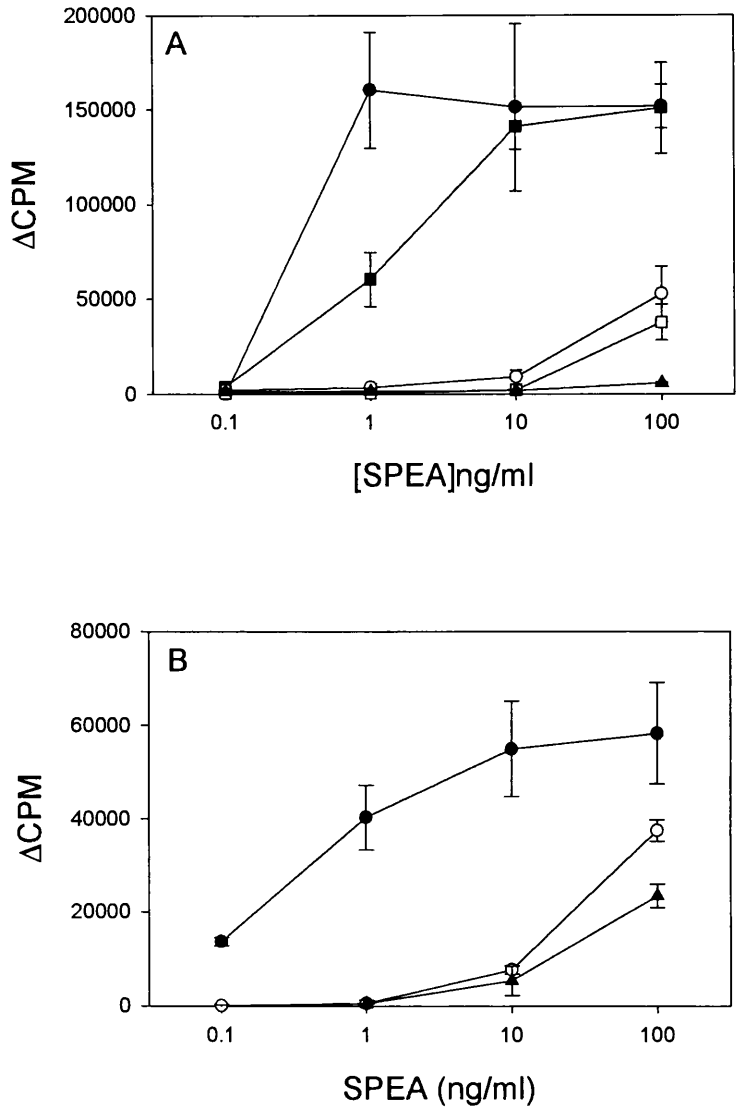


Fig. 5.1. Influence of HLA-DQ on DO11.10 splenocyte proliferation in response to SPEA presented by B-LCLs. **A)** two *HLA-DQA1*01* cell lines PGF (●) and WT46 (■), two *DQA1*05* cell lines, IDF (○) and TISI (□) and the class II negative cell lines BLS (▲). **B)** PGF (●) and TISI (□) and BLS (▲). Mean ΔCPMs +/- 1SD are shown.

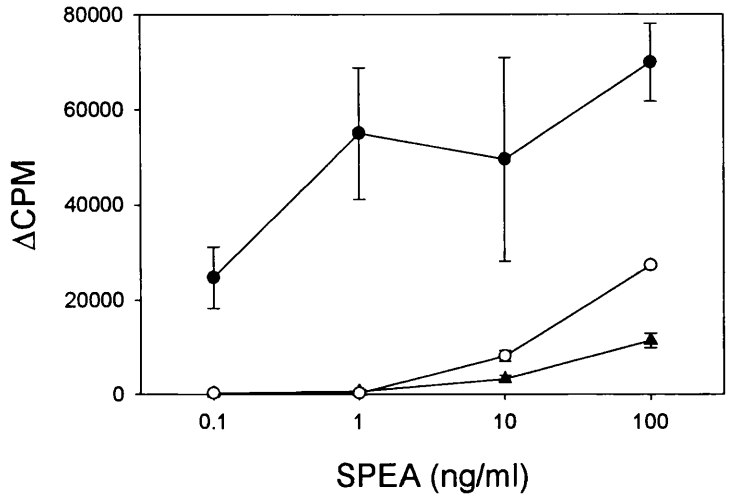


Fig. 5.2. Influence of HLA-DQ on DO11.10 splenocyte proliferation in response to SPEA presented by BLS transfectants. Proliferation in response to SPEA in the presence of HLA-DQ transfectants; *HLA-DQA1*0102 /DQB1*0602* (●), *HLA-DQA1*0301 /DQB1*0302* (○) and untransfected BLS cell line (▲). Mean ΔCPMs +/- 1SD are shown.

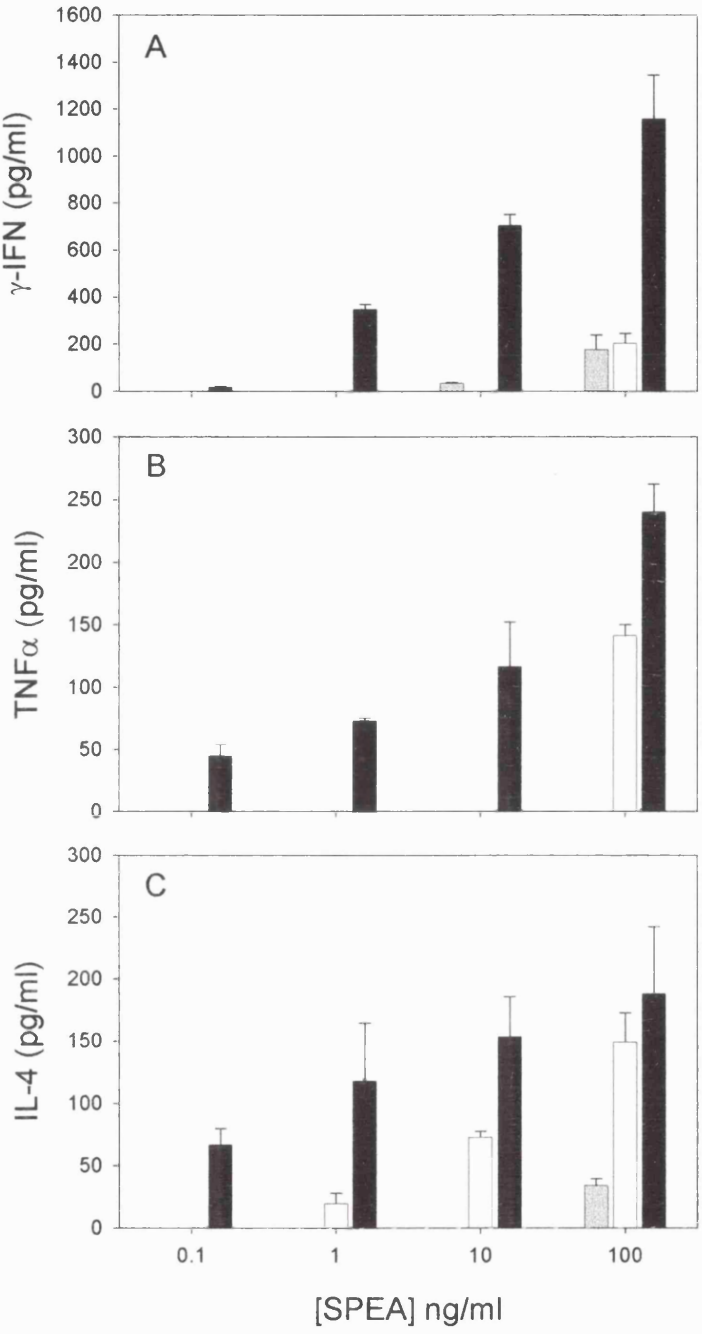


Fig. 5.3. DO11.10 splenocyte cytokine production in response to SPEA presented by B-LCLs. PGF (*DQA1*0102*) (■), IDF (*DQA1*0301*) (□), BLS (class II negative) (▒). **A)** γ-IFN, **B)** TNF-α, **C)**, IL-4. Bars show mean of three observations +/- 1 SD.

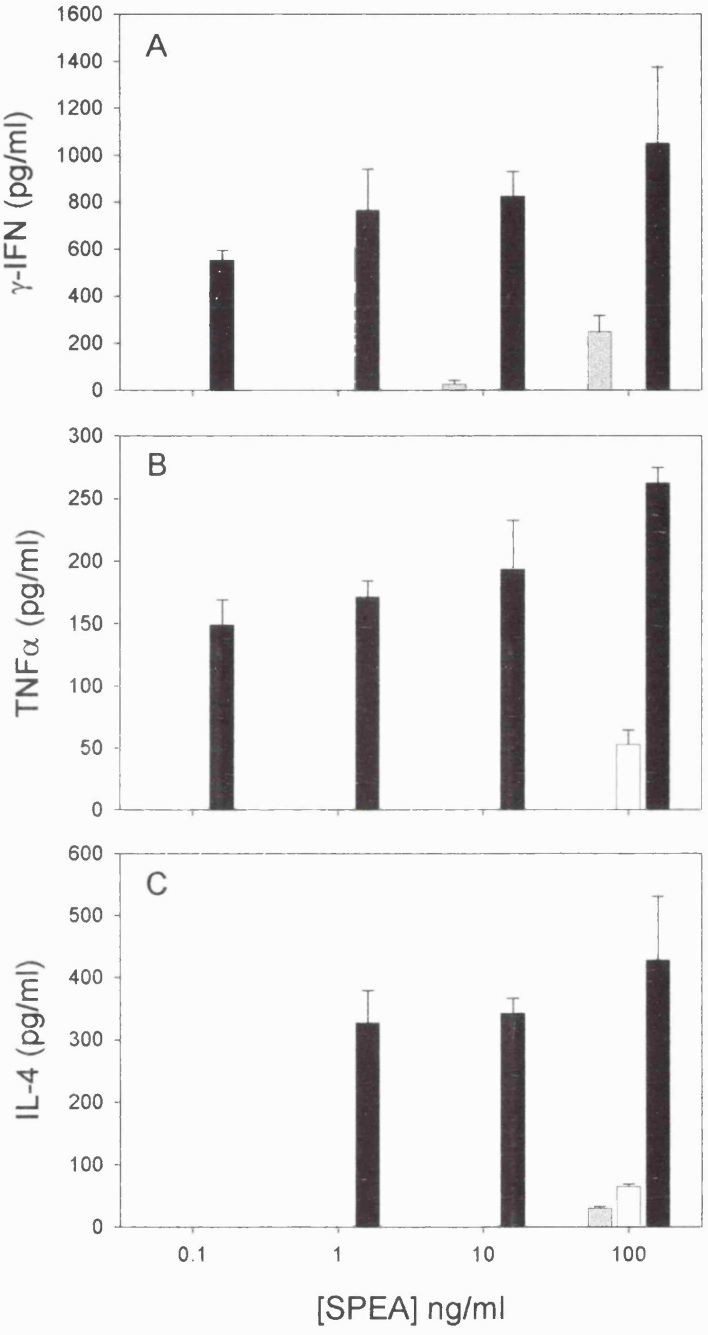


Fig. 5.4. DO11.10 splenocyte cytokine production in response to SPEA presented by BLS HLA–DQ transfectants. *HLADQA1*0102 / DQB1*0602* (■), *HLADQA1*0301 / DQB1*0302* (□), BLS (▒). **A)** γ-IFN, **B)** TNF-α, **C)**, IL-4. Bars show mean of three observations +/- 1 SD.

In each figure, the data are representative of two or more experiments performed.

Since elevated TNFα levels are of central importance in the lethality of superantigen mediated shock (Miethke, Wahl *et al.* 1992), it is noteworthy that, within the likely *in vivo* SPEA concentration range (<100ng/ml) (Sriskandan, Moyes *et al.* 1996), there was an absolute difference in the TNFα response, *DQA1*03/05* APCs eliciting no detectable TNFα release.

5.3.2 Influence of HLA-DQ on response of purified human T cells to SPEA

In this approach, purified human T cells selected for HLA class II negativity from healthy donor PBMCs, were used as responder T cells. SPEA was presented by B-LCLs in one of two ways.

Firstly, purified T cells were incubated with irradiated B-LCLs and SPEA at a range of concentrations. Successful class II depletion of T cells using the method described above was confirmed by FACS. One of three separate experiments is shown in figure 5.5 panels a-d. Additionally, as a control included each time the experiment was performed, it was demonstrated that class II negative T cells failed to proliferate in response to SPEA except in the presence of exogenous APCs. A representative result is shown (Fig 5.5e). High background counts attributable to alloreactivity were observed when purified T cells were incubated with exogenous APCs in the absence of any specific stimulus (Fig 5.5e). To counter the possibility that T cells activated through alloreactivity might express donor class II and so confound the observation of any differences in response attributable to exogenous class II, T cells were purified from an HLA-DQ3 homozygous donor. The rationale for this was that any T cell expressed class II would be of low SPEA-binding DQ3.

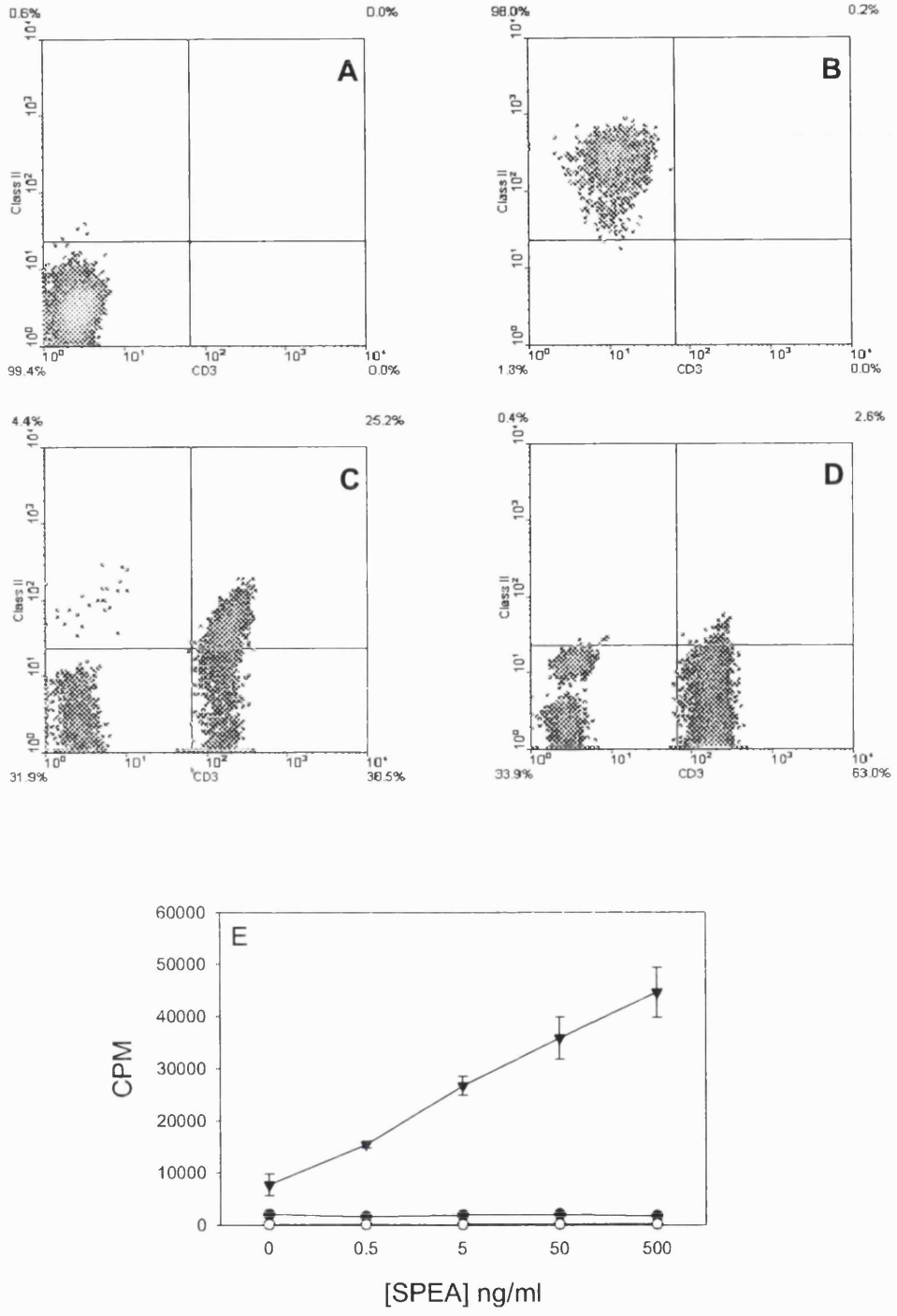


Fig. 5.5. Class II depletion of PBMCs. Demonstration of class II depletion by FACS **(A-D)** and response to superantigen stimulation **(E)**. **A)** second layer alone (negative control) **B)** PGF B-LCLs (positive control for class II) **C)** undepleted PBMCs **D)** class II depleted PBMCs. **E)** purified T cells fail to proliferate in the presence of SPEA either incubated alone (○) or with class II negative BLS cells (●) but show dose dependent proliferation when co-incubated with class II positive WT46 cells (▼).

In three comparisons of the response of purified T cells to SPEA in the presence of either *DQA1*05* or *DQA1*01* homozygous B-LCLs, SPEA-dose dependent proliferation was observed. Also, an effect of HLA-DQ on the magnitude of the T cell response was evident such that greater responses were seen in the presence of *HLA-DQA1*01* than in the presence of *DQA1*05*. In the representative experiment shown (Fig 5.6) the half maximal stimulation concentration for SPEA in the presence of *HLA-DQA1*01* was around 1 log lower than in the presence of *DQA1*05*. Stimulation index values are relatively low, because of the high background levels of proliferation.

To further counter the possibility that these high background counts could cause up-regulation of donor T cell class II, thus confounding observations related to class II expressed by exogenous APC, a second approach was adopted in which B-LCLs were pulsed with SPEA and fixed with paraformaldehyde prior to incubation with T cells. This served to remove any possibility that free SPEA might interact with HLA class II expressed on activated T cells. Interestingly, this had the additional benefit of lowering background proliferation to a fraction of that associated with the first approach. The level of stimulation was adjusted by altering the APC:T cell ratio. In the experiment shown (Fig. 5.7) comparing low and high binding HLA-DQ types, at APC: T cell ratios of 5:1 and 10:1 *DQA1*01* expressing APCs supported significantly higher levels of T cell activation than *DQA1*05* expressing cells ($P<0.05$ by t test).

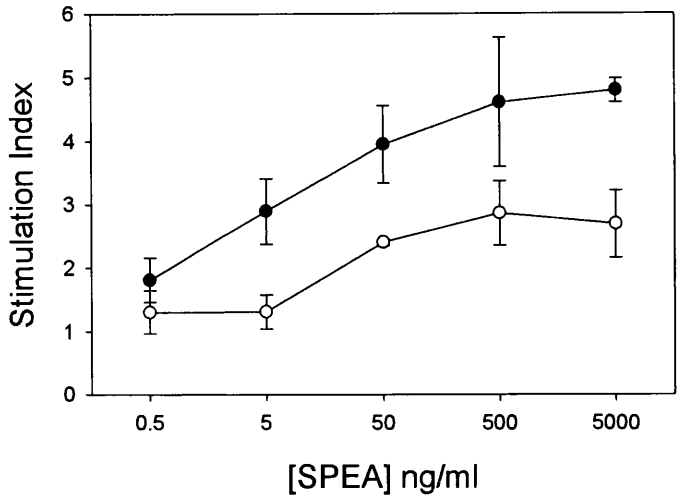


Fig. 5.6. Influence of HLA-DQ on proliferation of human T cells in response to SPEA. Irradiated B-LCLs used as APCs incubated with purified T cells and SPEA, *HLA-DQA1*01* homozygous APCs (PGF, WT46, SCHU) (●) and *HLA-DQA1*05* homozygous APCs (IDF and TISI) (○), means +/- 1SD are shown.

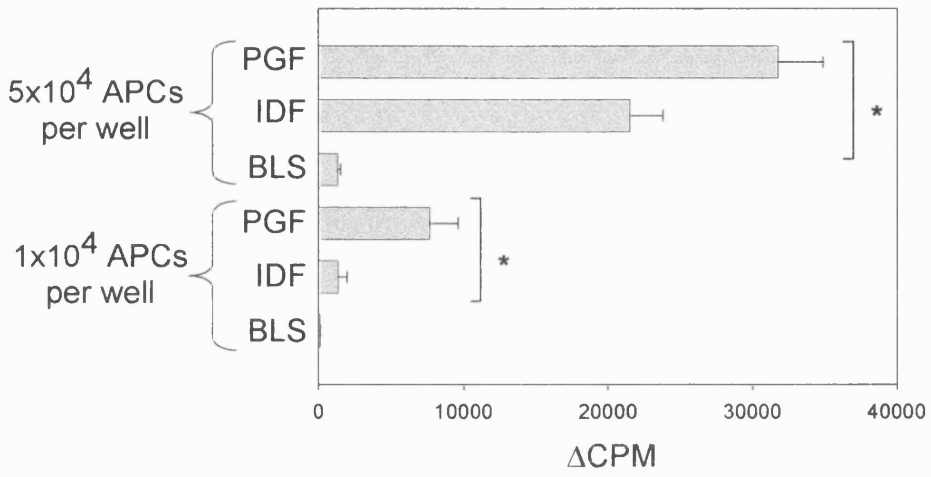


Fig. 5.7. Influence of HLA-DQ on proliferation of human T cells in response to SPEA. SPEA (1µg/ml) pulsed, paraformaldehyde fixed B-LCLs at two ratios of APC to T cell concentrations, $5x10^4$ APCs/well = 1 APC to 5 T cells, $1x10^4$ APCs/well = 1 APC to 10 T cells. Bars show mean +/- 1SD. * $P<0.05$ by t-test.

5.3.3 Influence of donor HLA class II on PBMC response to SPEA

In the third approach, in order to confirm whether an effect of HLA-DQ polymorphism remains apparent, notwithstanding other host factors which might be involved in the response to superantigens, PBMCs purified from HLA typed donors were stimulated with SPEA. Comparing three *HLA-DQA1*01* homozygous donors with three *HLA-DQA1*03/05* homozygous donors, no differences in proliferation were observed between the groups in the presence of medium alone or in response to PHA. However, the SPEA response was significantly greater for *HLA-DQA1*01* donors than *HLA-DQA1*03/05* donors at concentrations of SPEA ranging from 0.1-1000ng/ml (Fig. 5.8).

5.3.4 Influence of HLA-DR on presentation of SEA to purified human T cells

To extend these observations regarding the SPEA interaction with HLA-DQ to include my finding of differences in SEA binding to HLA-DR4 and DR11 we used the second of the three approaches described above. Irradiated B-LCLs homozygous for either *DRB1*0401* (WT51, BOLETH, PRIESS) or *HLA-DRB1*11* (IDF, TISI, SWEIG) were used to present SEA to T cells purified from a donor who was HLA-DR11/DR13. This donor was chosen on the basis of this HLA type since DR13 shares the β-chain D70Q polymorphism with DR11 (see table 3.2). At a lower concentration range than was used for SPEA, in keeping with the greater potency of SEA, a dose dependent proliferation above background was observed and higher levels of proliferation were observed with *DRB1*0401* than with *DRB1*11* (Fig. 5.9). No difference was seen between the HLA types in either background proliferation or PHA response.

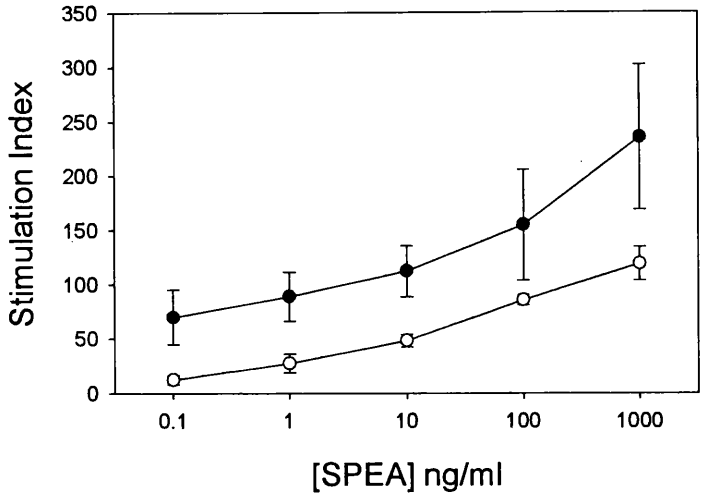


Fig. 5.8. Influence of donor HLA-DQ on PBMC proliferation in response to SPEA. *HLA-DQA1*01* homozygous donors (●) and *HLA-DQA1*03/05* donors (○). Means +/- 1sd are shown, n=3 in each group.

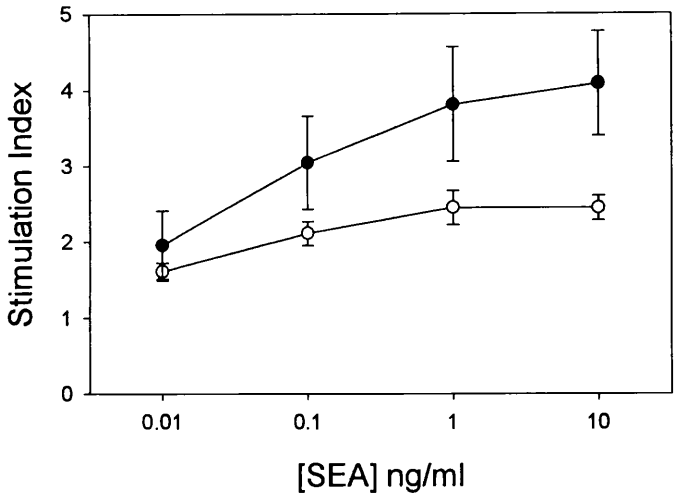


Fig. 5.9. Influence of HLA-DR on proliferation of human T cells in response to SEA. Irradiated B-LCLs used as APCs incubated with purified T cells and SPEA, *HLA-DRB1*0401* homozygous APCs (WT51, BOLETH, PRIESS) (●) and *HLA-DRB1*11* homozygous APCs (TISI, IDF, SWEIG) (○).

B) Influence of class II on the Vβ specific T cell response to superantigens

In order to study the influence of class II on the TCR Vβ repertoire of the T cell response to superantigen stimulation, the baseline characteristics of this response were defined *in vitro*. PBMCs were stimulated with SPEA at a range of concentrations and the Vβ repertoire of responding T cells analysed by flow cytometry at different time points.

5.3.5 Definition of the Vβ response to superantigen by flow cytometry

The forward scatter (FSC) and side scatter (SSC) properties of resting lymphocytes, in fresh unstimulated PBMCs, and blasting lymphocytes, involved in the response to superantigen or other stimulus, are markedly different. The proportion of blasts in a population of lymphocytes following stimulation will evidently depend on the magnitude of the response. Figure 5.10 illustrates how the FSC/SSC properties of lymphocytes following superantigen stimulation depends on the dose of superantigen used and how 'resting' and 'blasting' lymphocyte gates can be defined. The data presented in section A of this chapter demonstrate that the magnitude of proliferation which occurs following PBMC stimulation by superantigen is determined by HLA class II. Consequently if a comparison is to be made of the superantigen response in donors of different HLA type, assessment of the Vβ repertoire has to be confined to the blasting lymphocytes and not include cells falling into a resting lymphocyte gate. Gating on the entire lymphocyte population would skew the observed Vβ repertoire towards the resting T cell repertoire at low doses of stimulation or in donors of low responding class II type.

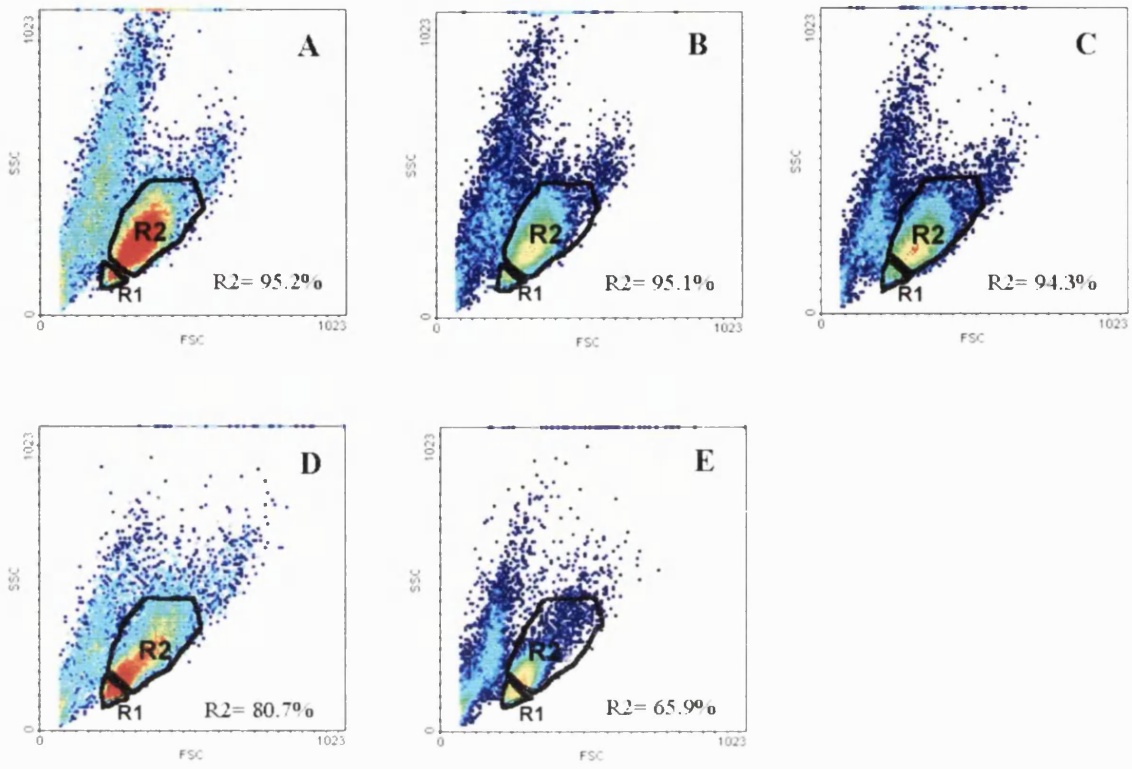


Fig. 5.10. Definition of 'resting' and 'blasting' lymphocyte gates. A) to **E)** Forward Scatter *vs*. Side Scatter plots of PBMCs stimulated with SEA at 1000, 100, 10, 1, 0.1ng/ml of SEA. The proportion of lymphocytes lying in the 'blasting' gate R2 falls from 95.2% in **A)** to 65.9% in **E)** as stimulation falls.

In order to determine whether stimulation of PBMCs by a non-specific stimulus is associated with any change in the TCR Vβ repertoire, FACS analysis was performed of Vβ against CD4 before (in a resting lymphocyte gate) and after (in a blasting lymphocyte gate) PHA stimulation. Using a panel of 18 Vβ antibodies no significant change in any of these Vβs was noted following PHA stimulation (Fig. 5.11). Furthermore, 48.7 +/- 2.4% and 54.0 +/- 4.18% of the total Vβ repertoire was accounted for in resting PBMCs and PHA stimulated PBMCs respectively (n=6), confirming that PHA stimulation does not cause significant skewed expansions of Vβ types not included in the panel. PHA and anti-CD3 have been used in this way as controls for superantigen stimulation in previous studies (Newton, Dohlsten *et al.* 1996).

In order to confirm that no differences exist in the PHA response between donors homozygous for *HLA-DQA1*01* and *DQA1*03/05,* PBMCs from six individuals; three in each group, were stimulated with PHA, harvested at 7 days and the Vβ repertoire of both CD4 and CD8 cells analysed. This FACS analysis using a panel of 21 Vβ antibodies was kindly performed by Dr. Daniel Douek, Vaccine Research Center, NIH, Bethesda, MD, USA. No statistically significant difference in the percentage of any TCR Vβ type was found. (Fig. 5.12).

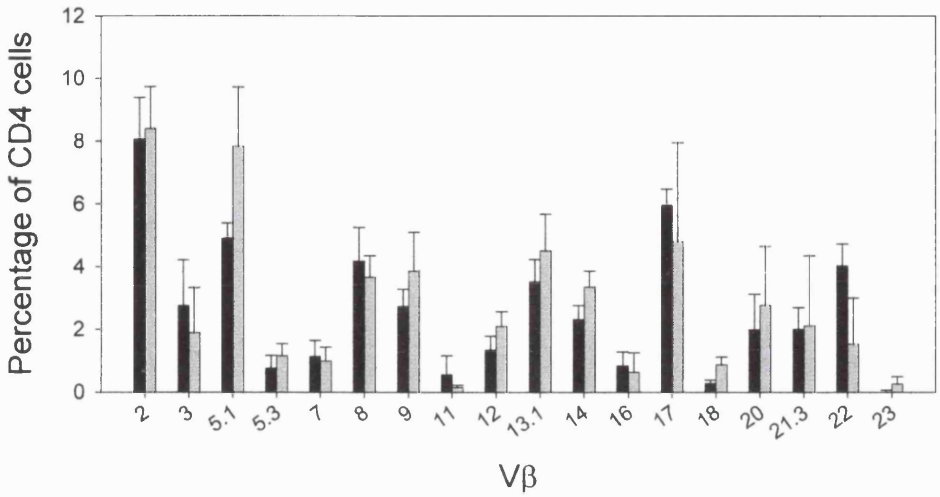


Fig. 5.11. Influence of PHA stimulation on T cell Vβ repertoire. Vβ percentages of CD4 positive lymphocytes in unstimulated PBMCs (■) and blasting lymphocytes following 7 days PHA stimulation (■). Bars show mean +/-1SD n=6.

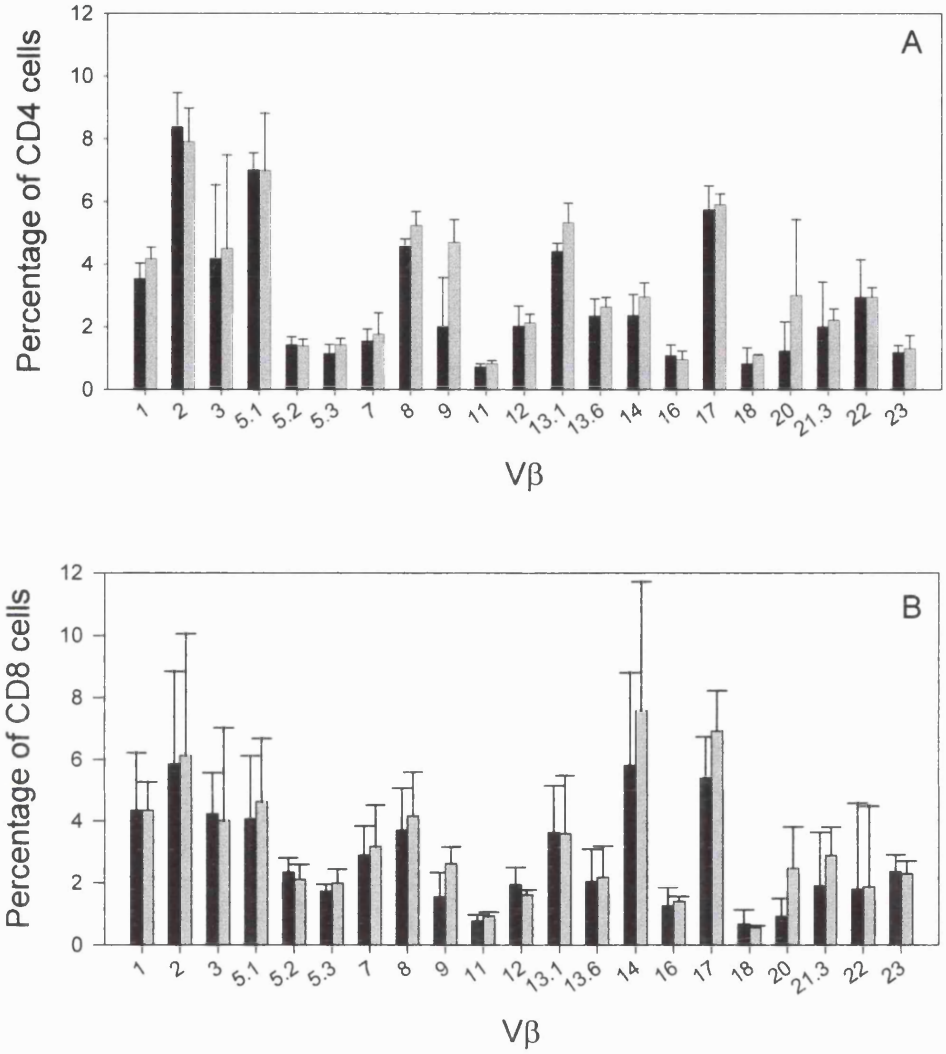


Fig. 5.12. Influence of donor HLA-DQ alpha chain type on Vβ repertoire following PHA stimulation of PBMCs. Bars show mean percentage +/- 1SD of total number of **(A)** CD4 or **(B)** CD8 cells falling inside a blasting lymphocyte gate following 7 days stimulation. *DQA1*01* (■) or *DQA1*03/05* (■) homozygous donors, n=3 in each group.

5.3.6 Vβ repertoire changes following SPEA stimulation.

PBMCs from six individuals; three homozygous *HLA-DQA1*01*, three HLA-DQ*A1*03/05* were stimulated with SPEA (100ng/ml) and the Vβ repertoire of blasts analysed after 7 days (Fig 5.13). Under these conditions, 73% of the Vβ repertoire was made up of Vβ14 (41.7+/- 10.3%), Vβ12 (19.5 +/- 4.2%) and Vβ13.1 (11.8 +/- 4.5%). The only other Vβs contributing more than 1% to the CD4 repertoire were Vβ3 (3.9 +/- 0.99%), Vβ20 (3.2 +/- 1.7%) and Vβ21 (2.39 +/- 1.2). Although under these conditions no statistically significant difference was observed for any Vβ between the two HLA-DQ types, it is noteworthy in the light of results from subsequent experiments that at this concentration the proportion of blasting CD4 cells made up of Vβ12 and 14 cells was higher in the *DQA1*03/5* donors while the Vβ13.1 response, which has not been described previously for SPEA, was more marked in the *DQA1*01* donors.

The concentration of superantigens which will occur *in vivo* during the course of an infection will inevitably vary with time, between different tissues and between individuals (Sriskandan, Moyes *et al.* 1996). In order to explore the possibility that SPEA concentration might differentially affect the response of different TCR Vβs, and that this might depend on HLA-DQ type, the Vβ changes following SPEA stimulation were studied in PBMCs from six individuals at SPEA concentrations ranging from 1 to 100ng/ml (Fig. 5.14). Vβ proportions of CD4 cells were assessed for Vβ2, 3, 5.1, 8, 12, 13.1 and 14 (selected on the basis of the results above) in three homozygous *HLA-DQA1*01* and three HLA-DQ*A1*03/05* individuals.

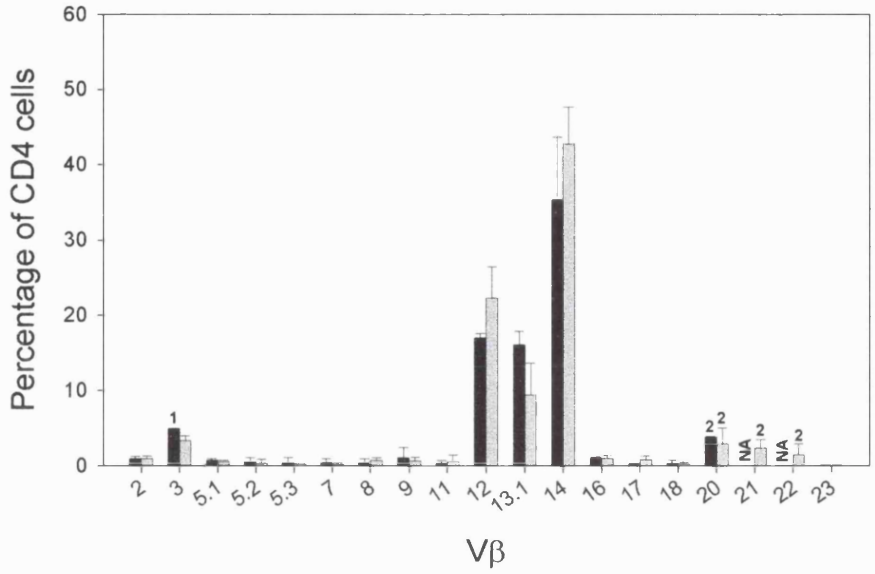


Fig. 5.13. Vβ repertoire following SPEA stimulation of PBMCs. Bars show mean percentage ± 1SD of total number of CD4 cells falling with in a blasting lymphocyte gate following SPEA 100ng/ml. *DQA1*01* and *DQA1*03/05* donors, n=3 in each group except [1]n=1, [2]n=2 and NA; not analysed.

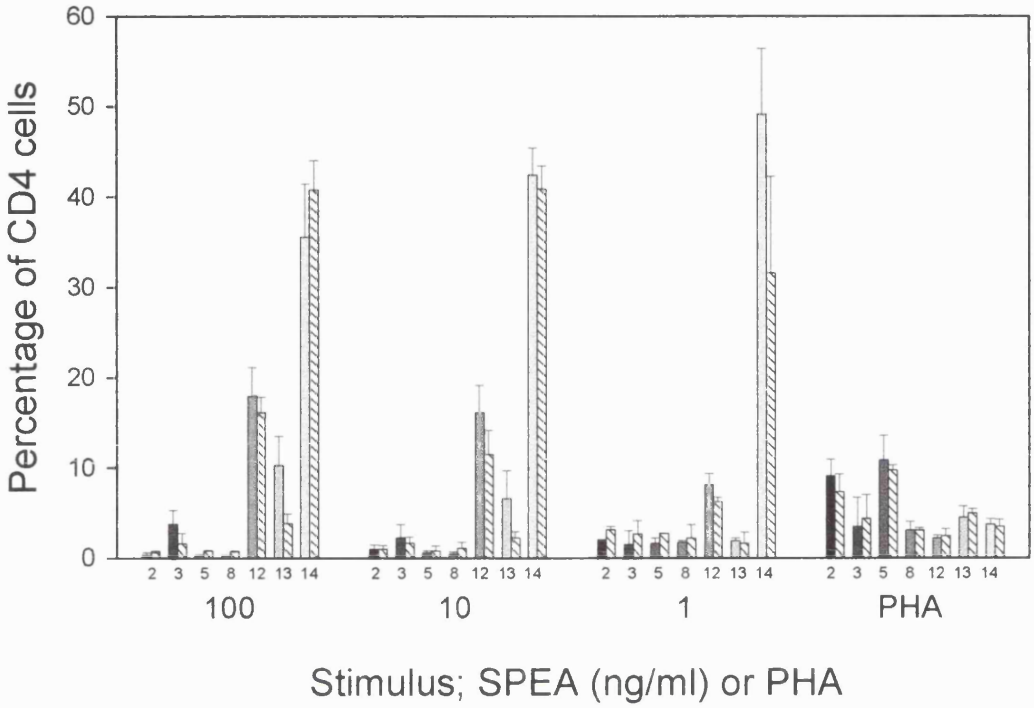


Fig 5.14. Influence of donor HLA-DQ on Vβ response to SPEA stimulation. Bars show mean percentage ± 1SD of total CD4 cells for *DQA1*01* homozygous ( ) and *DQA1*03/05* donors ( ) for seven different Vβs: 2, 3, 5.1, 8, 12, 13.1 and 14 reading left to right. N=3 in each group.

A concentration dependent increase in the proportion of Vβ12 cells was seen in both groups. In contrast the proportion of Vβ14 fell with increasing SPEA concentration in the *HLA-DQA1*01* donors and increased in the *DQA1*03/05* group. The proportion of Vβ13.1 cells increased with SPEA concentration to a greater extent in the *DQA1*01* donors than in the *DQA1*03/05* donors and this resulted in a statistically significantly higher proportion of Vβ13.1 in the *DQA1*01* donors at both 10 and 100ng/ml SPEA ($P<0.05$ in each case by t test). It is also noteworthy that while Vβs 2, 5.1 and 8 are effectively absent from the blasting T cell population at 10 and 100ng/ml SPEA, as in the previous experiment, a population of Vβ3 blasts remains and there is a trend towards this increasing as a proportion of the whole CD4 population as SPEA concentration rises, in the *DQA1*01* donors.

In view of the unexpected observation that superantigen concentration determines Vβ specific T cell expansion, the impact of SPEA dose on Vβ response was studied in detail. Figure 5.15 shows the relationship between Vβ repertoire and concentration of SPEA extended above 100ng/ml and below 1ng/ml for a single *HLA-DQA1*05* homozygous donor. It is apparent from this result that different Vβs are drawn into the superantigen response as the concentration rises. The Vβ specific response which is confined to Vβ14 below 1ng/ml spreads to include Vβ12 at a range between 1 and 100ng/ml and above this to include Vβ13.1 and then Vβ3. A generalised spreading to other Vβs is not seen with Vβ2, 5, and 8 all remaining below 0.2% of the total CD4 population even at 5000ng/ml.

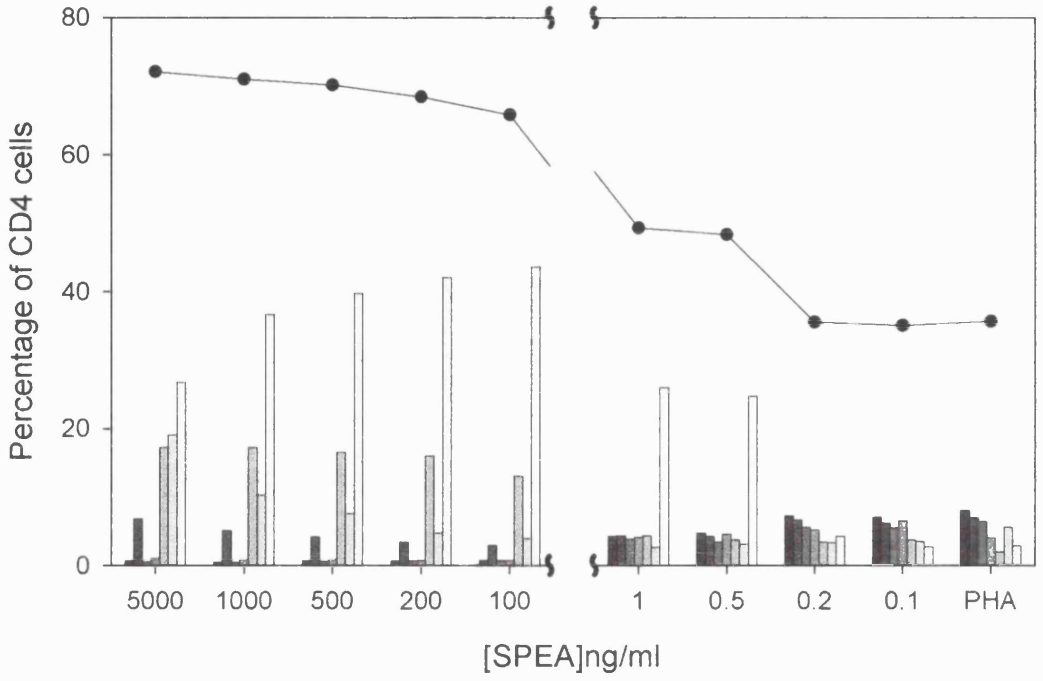


Fig. 5.15. Influence of SPEA dose on Vβ repertoire of T cell blasts. Results are from a single donor PBMCs stimulated with SPEA at concentrations above 100ng/ml and below 1ng/ml. Bars show percentage of total CD4 positive blasts for seven Vβs: 2, 3, 5,1, 8, 12, 13.1, and 14, reading left to right, at each SPEA concentration. Percentage of the total CD4 population accounted for by the seven Vβs analysed is also shown.

Two further donors, one *HLA-DQA1*01* and one *HLA-DQA1*05* homozygous, were then studied across a concentration range of 0.05 – 5000ng/ml. The same pattern of Vβ response is seen for these two donors in Fig 5.16. Specifically a rise in Vβ14 precedes rises in other Vβs, peaks as Vβ12 begins to rise and declines further as Vβ13.1 and Vβ3 begin to be drawn into the response. The striking difference seen here between these donors is that the concentration range at which the changes are seen is approximately 1 log lower in the *HLA-DQA1*01* donor (Fig 5.16a) than in the *DQA1*05* donor (Fig 5.16b).

In order to establish that differences in Vβ response to SPEA stimulation in these donors relates directly to differences in HLA class II presentation of SPEA, irradiated HLA homozygous B-LCLs were used to present SPEA to purified T cells from a single, HLA-*DQA1*05* homozygous, donor (Fig. 5.17). In the absence of any specific stimulus, a trend towards slightly lower Vβ2 and 13.1 and slightly higher Vβ8 percentages was noted following T cell incubation with *DQA1*01* homozygous B-LCLs, however all of the seven Vβs analysed were found in proportions similar to unstimulated PBMCs irrespective of B-LCL HLA type. The effect of SPEA on the Vβ repertoire of T cell response was markedly different in the presence of *HLA-DQA1*01* APCs than in the presence of *HLA-DQA1*05* APCs. Furthermore the differences observed match closely with the differences seen comparing the PBMC response to SPEA in *HLA-DQA1*01* and *DQA1*03/05* donors. Looking at Vβ14 and Vβ12, which are the main Vβs targeted by SPEA, in the presence of *HLA-DQA1*01* APCs the proportions of these Vβs are higher at the lower concentration of SPEA; Vβ14 then declines as the SPEA dose rises.

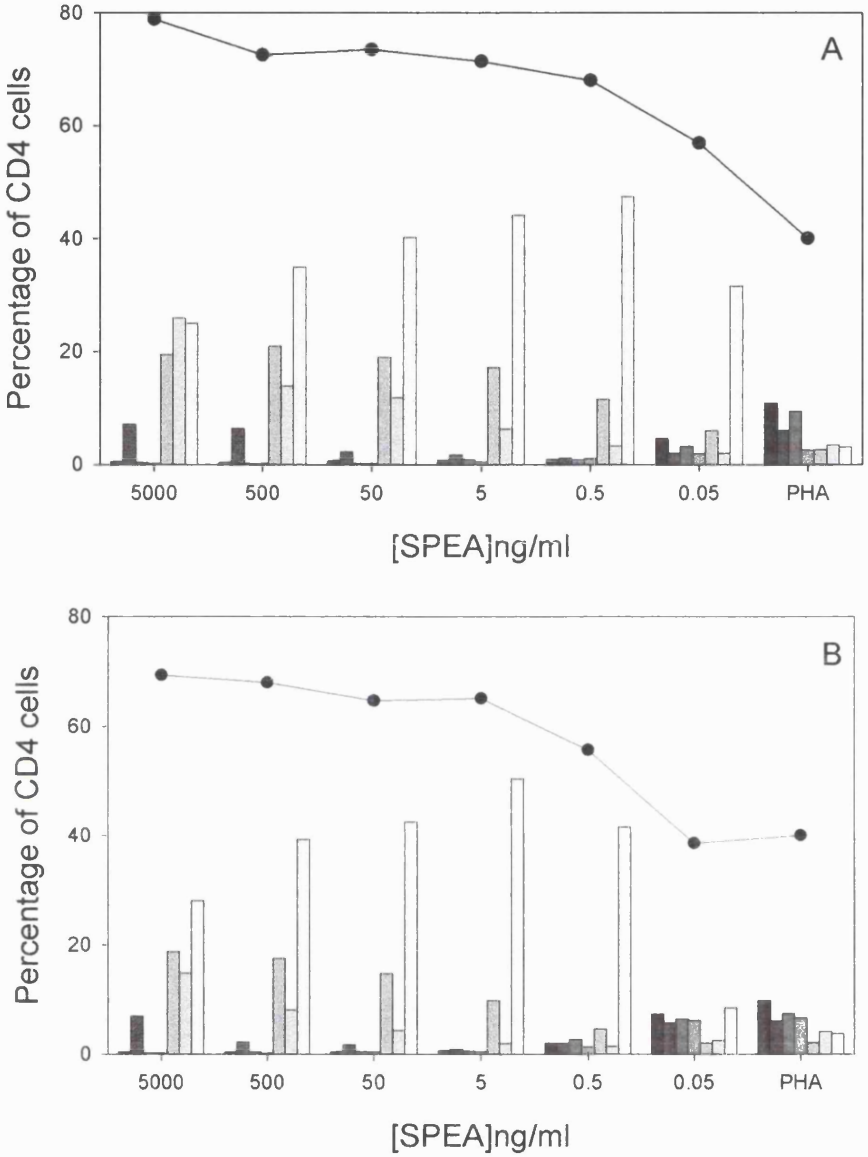


Fig. 5.16. Influence of SPEA dose on Vβ repertoire of T cell blasts. Results from two individuals are shown. A) *HLA-DQA1*01* homozygous donor. B) *HLA-DQA1*05* homozygous donor. Bars show percentage of total CD4 cells for seven Vβs; 2, 3, 5, 8, 12, 13.1 and 14 reading left to right. Total percentage of the CD4 population accounted for by the seven TCR Vβs analysed is also shown.

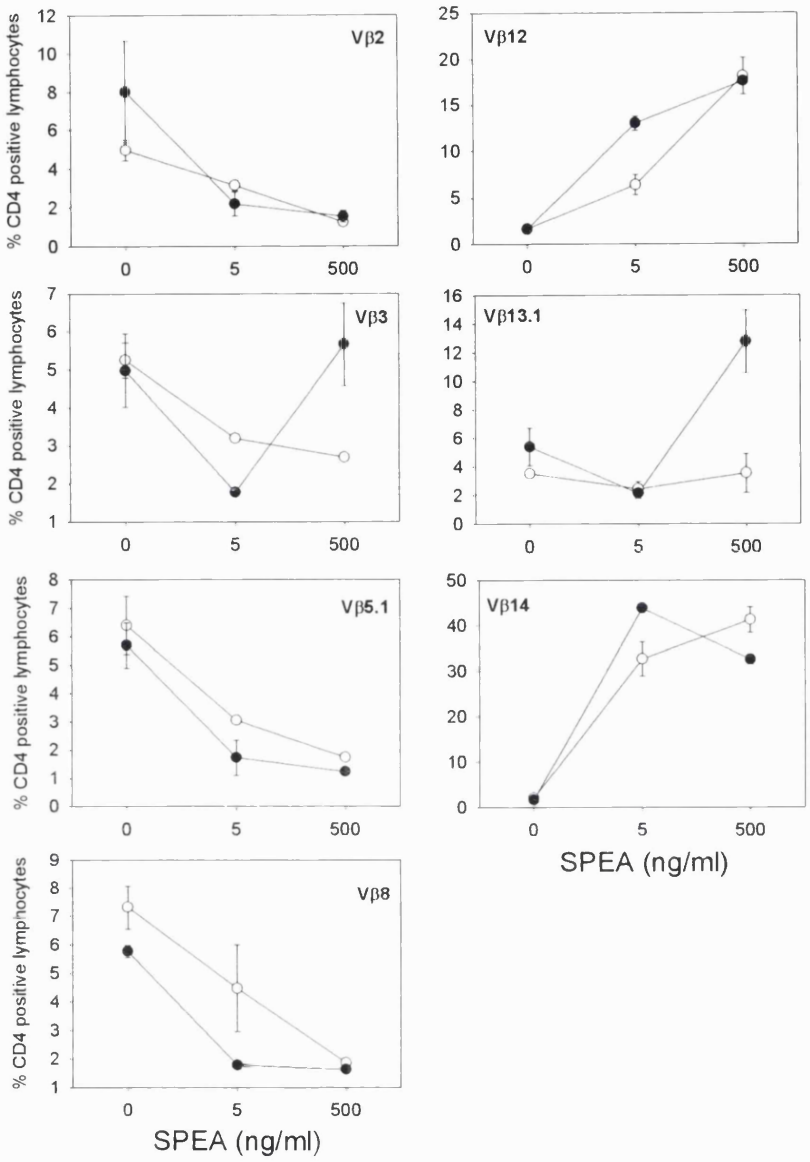


Fig. 5.17. Influence of APC HLA-DQ on Vβ repertoire of T cell response to SPEA. Each graph shows, for a particular Vβ, the mean percentage ± 1SD of total CD4 positive blasts following incubation of purified T cells with B-LCLs; *DQA1*01* homozygous (●) n=3 or *DQA1*05* homozygous (○) (n=2) and either medium alone, or SPEA at 5 and 500ng/ml.

Looking at Vβs 2, 5.1 and 8, which are not targeted by SPEA, the relative contribution made by these Vβs to the CD4+ T cell repertoire in response to SPEA is small and diminishes as SPEA concentration rises. Furthermore, in the presence of *DQA1*01* APCs, the relative contribution made by these Vβs is lower at the 5ng/ml SPEA concentration. Finally, looking at the Vβs which are drawn into the SPEA response at higher concentrations, Vβ13.1 and 3, the contribution made to the total CD4 population by these Vβs rises at the higher SPEA concentration in the presence of *DQA1*01* APCs but not in the presence of *DQA1*05* APCS. In summary, the Vβ response to SPEA in T cells from an HLA-*DQA1*05* homozygous donor can be made to resemble the Vβ response of HLA-*DQA1*01* homozygous donors by addition of *HLA-DQA1*01* homozygous APCs.

5.3.7 Vβ repertoire changes following SEA and SEB stimulation

In view of the unexpected finding that SPEA concentration determines not only the magnitude of the T cell response but also its Vβ specificity, the relationship between concentration and Vβ repertoire was explored for two important staphylococcal superantigens, SEA and SEB. The Vβ response to SEB has been reported previously as targeting Vβs, 3, 12, 14, 15, 17, 20 (Marrack and Kappler 1990). Analysis of the relationship between SEB concentration and Vβ response are shown for two donors in figures 5.18 and 5.19. In the first experiment (Fig 5.18) analysis of four Vβs targeted by SEB; Vβ 3, 12, 14, 17 and six others; Vβ1, 2, 5, 8, 13, 22 is shown. At the lowest concentration marked response of Vβs 3, 12, 14, and 17 is apparent and as the concentration rises, Vβs 1, 5, and 22 are drawn into the response while Vβs 2, 8 and 13 remain undetectable. It is also striking that while

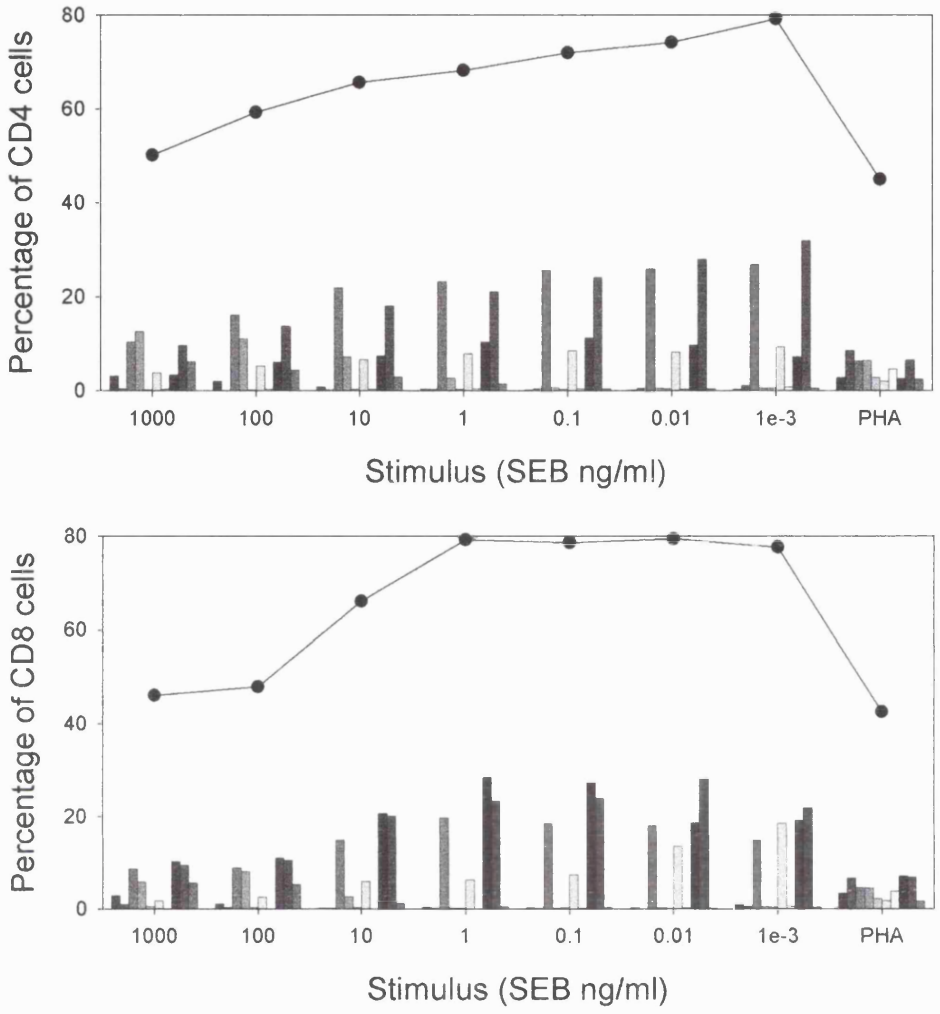


Fig. 5.18. Influence of SEB dose on Vβ repertoire of T cell blasts. Results from a single individual are shown. Bars show percentage of total CD4 or CD8 cells for 10Vβs; 1, 2, 3, 5, 8, 12, 13.1, 14, 17, 22 reading left to right. Total percentage of the CD4 population accounted for by the 10 Vβs analysed is also shown.

100% of the CD4 and CD8 populations are made up to Vβ 3, 13, 14, 17 at lower concentrations, only 50% are accounted for at the highest concentrations by the panel of Vβ antibodies used, implying that other Vβ expansions are taking place at higher concentrations. In a second donor (Fig. 5.19), a wider panel of Vβs was studied at the highest concentrations; Vβs 1, 2, 3, 4, 5.1, 8, 9, 11, 13.1, 14, 17, 20, 22 and 23. Also concentrations below 1pg/ml were studied focusing on Vβs 1, 3, 5, 12, 14, and 17. As in the first donor, Vβs 3, 12, 14, and 17 were found to be the main responding Vβs with Vβs 1, 5 and 22 drawn in at the highest concentrations. Additionally Vβs 4 and 20 were found to respond at high SEB concentrations. At the lowest concentrations studied the Vβ specific stimulation of Vβ12 and 14 was lost below 1pg/ml and of Vβ3 and 17, below 10fg/ml.

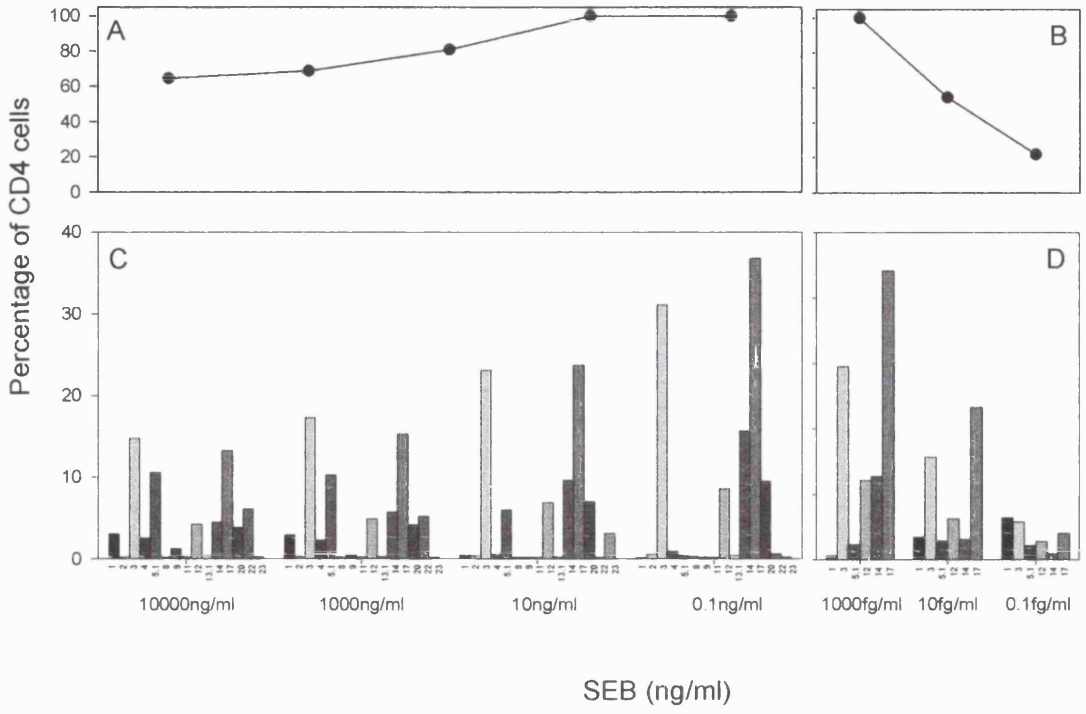


Fig. 5.19. Influence of SEB dose on Vβ repertoire of T cell blasts. Results from a single individual are shown. Bars show percentage of total CD4 cells. **C)** Vβs 1, 2, 3, 4, 5.1, 8, 9, 11, 12, 13.1, 14, 17, 20, 21 and 22 and **D)** Vβs1, 3, 5.1, 12, 14 and 17 are shown reading left to right. Total percentage of the CD4 population accounted for by the Vβs analysed is also shown in **A)** and **B)** (–●–).

The Vβ response to SEA has been reported previously as targeting Vβs 1, 5.2, 6, 7, 9, 18 and 22 (Newton, Dohlsten *et al.* 1996). Analysis of the relationship between SEA concentration and Vβ response are shown for two donors in figure 5.20. Ten Vβs were analysed including three of those known to be targeted in the SEA response – Vβ1, 9 and 22, and eight others; Vβ2, 3, 5.1, 8, 17, 20, and 23. In both donors, response of Vβ22 is apparent at the lowest concentration of 0.1pg/ml. Vβ9 and Vβ1 responses are at around 10pg/ml and 100pg/ml respectively with Vβ5.1 and 8 being drawn into the response at higher concentrations. No response from Vβ3, 17, 20 or 23 is seen even at the highest concentration of 100ng/ml. In order to determine whether HLA-DR polymorphisms might result in differences in Vβ repertoire following SEA stimulation, T cells purified from a single HLA-DR11/13 donor were stimulated with SEA at 10 and 1pg/ml or medium alone in the presence of HLA homozygous *DRB1*0401* (PRIESS, BOLETH, WT51) or *HLA-DRB1*11* (IDF, TISI, SWEIG) B-LCLs. The Vβ repertoire was analysed for six Vβs, two targeted by SEA (Vβ22 and 9) two involved in the SEA response at high concentrations (Vβ1 and 5.1) and two not targeted by SEA (Vβ2 and 3) (Fig. 5.21). A marked expansion of Vβ22 was observed in response to SEA presented by both HLA-DR4 and DR11 but was greater at the lower concentration, 1pg/ml, when presentation was by DR4. Similarly the Vβ9 response to SEA was greater in the context of DR4 presentation. As expected, the relative contribution of Vβ2 and 3 to the CD4+ T cell population following SEA stimulation was small. However, a trend towards a expansion of both Vβ1 and 5 was found. Comparing the Vβ repertoires at 10 and 1pg/ml, in the context of DR4 the Vβ1 response was greater at 10pg/ml than 1pg/ml.

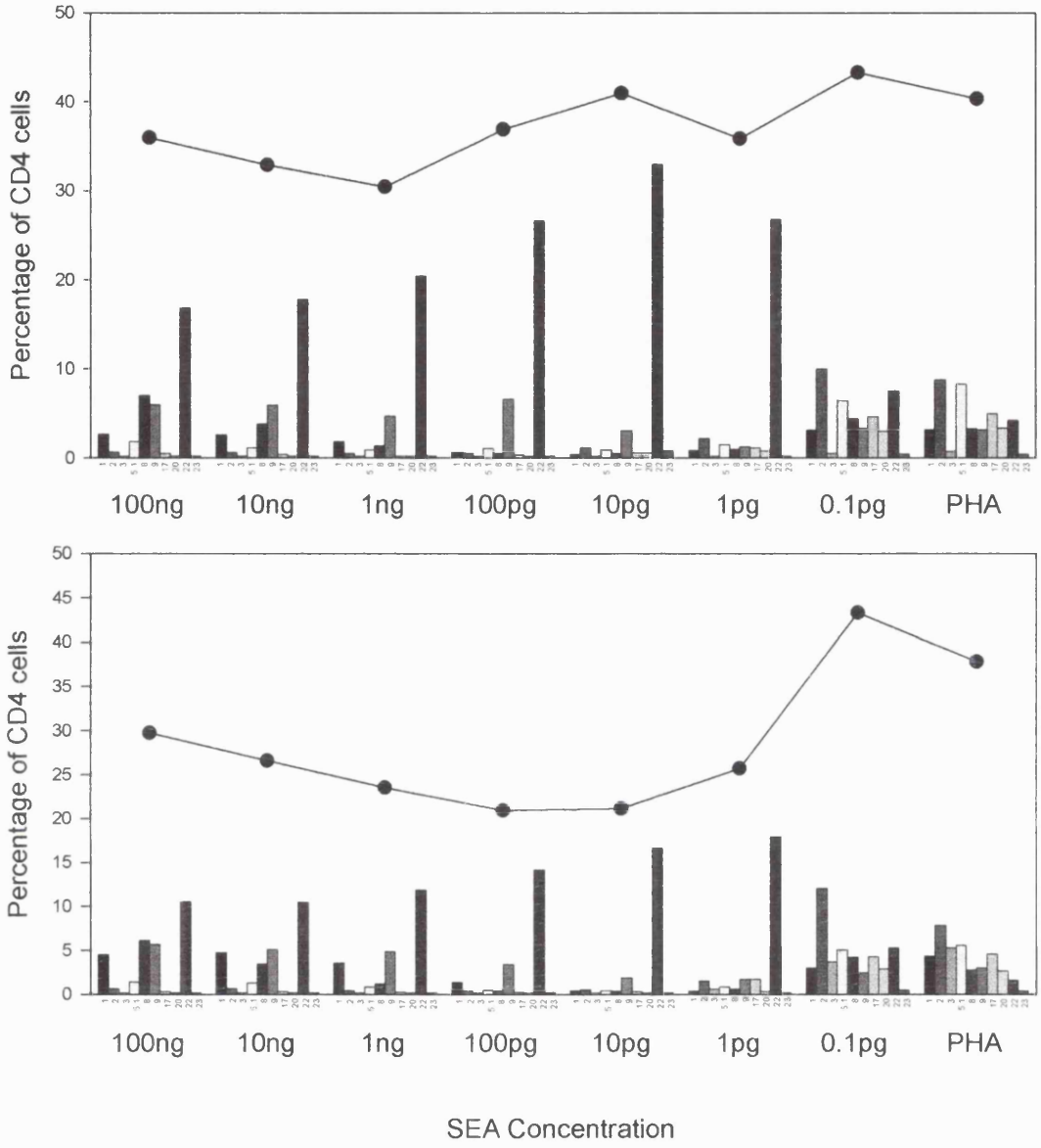


Fig. 5.20. Influence of SEA dose on Vβ repertoire of T cell blasts. Results from two individuals are shown. Vβs 1, 2, 3, 5.1, 8, 9, 17, 20 22 and 23 are shown reading left to right. Bars show percentage of total CD4 cells. Total percentage of the CD4 population accounted for by the Vβs analysed is also shown (–●–).

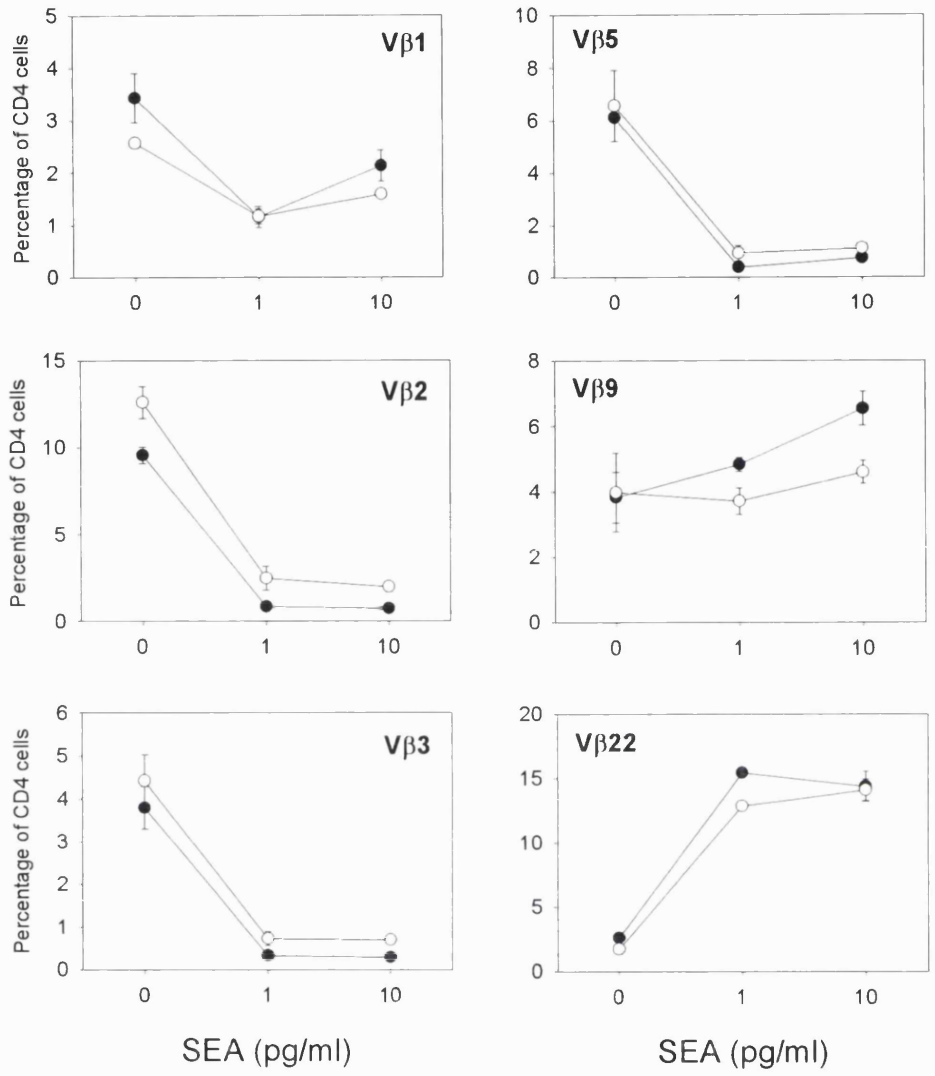


Fig. 5.21. Influence of APC HLA-DR on Vβ repertoire of T cell response to SEA. Each graph shows, for a particular Vβ, the mean percentage ± 1SD of total CD4 positive blasts following incubation of purified T cells with B-LCLs; *DRB1*0401* homozygous (●) n=3 or *DRB1*11* homozygous (○) (n=3) and either medium alone, or SEA at 0.1 and 1ng/ml.

5.4 Discussion

A) Influence of class II on the magnitude of the T cell response to superantigens

The data presented here demonstrate that the differences in binding of SPEA by different HLA-DQ alleles described previously are associated with differences both in the magnitude and the quality of the T cell response. The three approaches used were selected to provide different levels of control for potentially confounding issues associated with the responding T cells. DO11.10 splenocytes represent an ideal readout of SPEA presentation by HLA class II. Although these mice were developed for the study of atopy, they are transgenic for mVβ8.2 and virtually all their circulating lymphocytes express this TCR (Murphy, Heimberger *et al.* 1990). Murine *TRBV13-2,* which encodes mVβ8.2, shows the closest nucleotide sequence homology to the human TRBV genes which encode hVβ14 and 12, the targets of SPEA in man (Wilson, Lai *et al.* 1988). At an amino acid level there is marked homology between mVβ8.2 and hVβ12 and 14 at sites of suggested SPEA binding (Sundberg, Li *et al.* 2002), which explains why mVβ8.2 is targeted in the murine T cell response to SPEA. Because of the inability of murine MHC class II to present SPEA except at high concentrations, it is reasonable to assume that the DO11.10 response to SPEA in the presence of HLA class II expressing APCs is due to presentation by HLA class II. The failure of DO11.10 splenocytes to proliferate in response to SPEA in the absence of exogenous HLA class II expressing cells confirms this. Consequently, the differences in both proliferation and cytokine responses to SPEA can only be attributable to differences in HLA class II. The finding of an IL-4 response to SPEA is at odds with previous observations that the T cell response to superantigens is

heavily Th1 biased (Schlievert 1993). This disparity is probably because DO11.10 mice, due to their BALB/c genetic background, are particularly susceptible to induction of a heavily Th2 skewed cytokine response. Given the suggested central role for TNFα in the pathogenesis of superantigen mediated shock, another striking feature of these data is the absence of any detectable TNFα response at SPEA concentrations below 100ng. In most tissues, outside the focus of infection itself, concentrations of SPEA *in vivo* are likely to be below this level (Sriskandan, Moyes *et al.* 1996).

The use of purified human T cells as a readout of superantigen presentation by class II has the obvious advantage that it allows more normal TCR engagement with HLA class II and the possibility of assessing T cell receptor repertoire changes. This methodology has been used widely in the past (Lavoie, Thibodeau *et al.* 1997; Kotb, Norrby-Teglund *et al.* 2002). One problem we encountered with this approach, demonstrated in Fig. 5.5e, was the high background proliferation, attributable to alloreactivity (Sherman and Chattopadhyay 1993), found when purified T cells were incubated with irradiated B-LCLs in the absence of any specific stimulus. The implications of this are two fold. Firstly the high background counts themselves make interpretation of differences between APCs difficult. Secondly, expression of donor HLA class II on T cells activated by alloreactivity raises the possibility that superantigen presentation by donor class II might obscure differences attributable to class II on exogenous APCs. I overcame some of these problems by fixation of superantigen – class II binding using paraformaldehyde. This approach has previously been demonstrated to disrupt presentation to T cells of conventional antigen but not

superantigens (Uchiyama, Imanishi *et al.* 1989). Nevertheless, notwithstanding the high backgrounds seen with irradiated APCs, differences in magnitude of the T cell response to SPEA presented by *HLA-DQA1*01* and *DQA1*03/05* were apparent with both approaches.

Numerous polymorphisms in immune response genes might be expected to determine the magnitude of response of individual donor PBMCs to a superantigen. For example, polymorphisms in the class II or cytokine promoter regions or in the intracellular signalling pathway. The results shown here demonstrate that differences in HLA-DQ binding of SPEA are associated with differences in both magnitude and quality of the T cell response despite the potential confounding effects of polymorphisms elsewhere in the immune response. In the absence of *in vivo* models of superantigenicity, in which HLA class II could be adequately controlled, these data represent the best evidence available for inter-individual differences in class II being associated with differential susceptibility to superantigens.

To extend these observations to include the differences identified in SEA binding to DR4 and DR11 only the second of the three approaches was feasible. SEA does not target mVβ8.2 and so DO11.10 splenocytes would not be appropriate responder cells. Mice expressing other, possibly human, TCR transgenes could have been utilised had such been available. Individuals homozygous for HLA-DR are much less common than HLA-DQ α-chain homozygotes, especially since the subtle differences identified between *HLA-DRB1*0401* and **0402* would make precise typing and matching essential. HLA-DR homozygous B-LCLs were used to present SEA to purified T cells, from a donor expressing HLA-DR alleles of probable low SEA binding (HLA-

DR11 and 13). SEA induced T cell proliferation was greater in the presence of DR4 than DR11 indicating that HLA-DR β-chain polymorphisms determine the T cell response to SEA just as HLA-DQ α-chain polymorphisms do to SPEA. It is worth emphasising that these two superantigens exemplify the two principal modes of superantigen interaction with the class II molecule; SPEA though a low affinity α-chain binding site and SEA through a high affinity β-chain binding site. The implication of this is that it is likely that all superantigens, with the possible exception of those which bind principally at the non-polymorphic DR α-chain, are subject to similar HLA class II influence.

B) Influence of class II on the Vβ response to SPEA.

Vβ specific change in T cell repertoire is a *sine qua non* of superantigenicity. Such changes are often regarded as evidence that a disease has a superantigen aetiology; Kawasaki's Disease is a prime example of this. The observation of variable changes in Vβ repertoire in diseases such a Kawasaki's has been interpreted as meaning that the disease may be the result of multiple different superantigens. However, even in toxic shock syndrome, attempts to define the Vβ specific changes seen in clinical samples have produced conflicting results (Michie, Scott *et al.* 1994; Wantanabe-Ohnishi, Low *et al.* 1995). An alternative explanation for these observations might be that Vβ specific changes are not a fixed property of an individual superantigen but determined by factors including concentration at the site of T cell activation and the HLA context in which the superantigen is acting. A relationship between superantigen/class II interactions and Vβ specific T cell response has been reported previously (Newton, Dohlsten *et al.* 1996). Using mutations of SEA targeted at the

class II α and β-chain binding sites, altered mitogenicity and TCR Vβ repertoire were observed.

Studies aimed at defining the Vβs targeted by individual superantigens *in vitro* have yielded very conflicting results. The *in vitro* Vβ specific targets of SPEA have been reported in six previous studies outlined in table 5.1. In every study to have looked for Vβ 12 and 14 changes, expansion of these two Vβ types has been found. The expansion of Vβ8, reported by Tomai *et al* and Braun *et al* was probably due to contamination of purified SPEA with other superantigens, in particular SMEZ, which characteristically stimulates Vβ 2, 4 and 8 (Tomai, Schlievert *et al.* 1992; Braun, Gerlach *et al.* 1993). Indeed Braun *et al* only found expansion of Vβ8 with purified, not recombinant SPEA. The finding of Vβ2 expansion with recombinant toxin has also not been a universal finding. Braun found no expansion of Vβ2 with recombinant SPEA. While Klein *et al* found Vβ2 expansions with SPEA, they observed that multiple mutations of SPEA resulted in loss of Vβ12 and 14 stimulating activity while only one of the 19 mutants had reduced Vβ2 stimulating activity (Kline and Collins 1997).

Table 5.1. Studies reporting the *in vitro* human Vβ targets of SPEA.

Study	Vβs identified	Method	Notes
(Imanishi, Igarashi *et al.* 1990)	8.2	FACS	No Abs to Vβ2, 12, or 14, but 80% of responding cells were Vβ8.2
(Abe, Forrester *et al.* 1991)	8, 12, 14	PCR	
(Tomai, Schlievert *et al.* 1992)	2, 8, 12, 14, 15	PCR	Expansions of Vβ8 and 2 were only marginal
(Braun, Gerlach *et al.* 1993)	2, 8, 12	FACS (gated on resting and blasting gates)	Expansion of Vβ2, but only with purified SPEA
(Kline and Collins 1997)	2, 12, 14	FACS	
(Fleischer, Necker *et al.* 1996)	12, 14	T cell hybridomas	Found no response to Vβ13.1 or 3

Of these studies, only Fleisher *et al* specifically sought Vβ13.1 and 3 response to SPEA. It is striking that the Vβ13.1 hybridoma used in this study responded to none of the superantigens against which they were tested, raising the possibility that it may have been unable to mount a superantigen response. The highest concentration used to stimulate the hybridomas with SPEA was 1000ng/ml and the APCs used (RAJI) express *DQA1*0501*. The failure to detect Vβ3 responses under these conditions is in keeping with the data presented here where Vβ3 responses were found only in purified T cells in response to SPEA presented by *DQA1*01* APCS and in only in

PBMCs from either HLA-DQ*A1*03/05* donors at 5000ng/ml or *DQA1*01* donors at above 500ng/ml.

Some of the concentrations of superantigen used in the work presented here are higher than have been used previously. However, concentrations of superantigen which are likely to occur during the course of infection undoubtedly vary widely. Regulation of superantigen expression is growth-phase dependent and there is a relationship between potency and regulation of expression such that the most potent superantigens such as SMEZ are only produced at very low concentrations (Unnikrishnan, Cohen *et al.* 1999; Gerlach, Fleischer *et al.* 2000). For less potent superantigens such as SPEA, concentrations have been measured clinically in streptococcal toxic shock at up to 1μg/ml (Sriskandan, Moyes *et al.* 1996). In a murine model of streptococcal necrotising fasciitis, circulating SPEA concentrations of 1 - 5μg/ml have been reported with concentration at the site of infection being up to five times the serum level (Sriskandan, Moyes *et al.* 1996). In contrast, concentrations at sites distant from infection, such as in lymphoid tissue may be much lower. While the lower limits of concentration at which superantigens may exert a biological effect *in vivo* have necessarily not been defined, it is reasonable to extend the concentration range over which the properties of superantigens are studied *in vitro* down to the lowest concentrations at which a biological effect can be identified.

There is considerable agreement between the Vβ repertoire observations described here for SEB and those in the literature. The main responding Vβs I have identified, Vβ17, 12, 14, and 3 are those identified by Fleisher *et al.* Of the Vβ identified here as responding only at high concentrations, Vβ20, 5.1, 1, 9 and 22, two, Vβs 1 and 9

were found to respond to SEB by Fleisher *et al.* and additionally Vβ20 was reported to expand by Kappler *et al.* (Kappler, Kotzin *et al.* 1989). Similarly the pattern of SEA response described here accords well with that of Fleisher *et al* with Vβ9, 22, 1 5.1 and 8.1 reported by both studies. Vβ16 reported to respond to SEA by Fleisher *et al* was not included in the panel analysed here.

In trying to define what features of TCR Vβ structure might determine these different superantigen Vβ repertoires the first striking correlation is the pattern of genetic relatedness which exists at a nucleotide level between *TRBV* families (Fig. 5.22) (Arden, Clark *et al.* 1995). The SPEA responsive Vβs 12 and 14 are closely related to each other and to the Vβs identified here as responding to SPEA at high concentrations; Vβ13.1 and Vβ3. Similarly, the core SEB responding Vβs also lie in this region of the TRBV dendrogram; Vβs, 3, 12, 14, 17. Several of the Vβs identified here as responding to high SEB concentrations are closely related to each other for example Vβ1, 5, 22. Similarly the Vβs reported as responding to SEA here and in Fleisher's study lie predominantly in the lower right branches of the dendrogram (Fig. 5.22). While this indicates that some sequence or structural features of the TCR are likely to determine whether it will respond to a particular superantigen, clearly there are numerous exceptions to the relationship between genetic relatedness and Vβ response.

The sites at which SPEA and SEB bind the Vβ region of the TCR have now been precisely defined for the interaction with mVβ8.2 (Li, Llera *et al.* 1998; Sundberg, Li *et al.* 2002). The sites overlap very extensively and this undoubtedly explains the overlapping Vβ specificities of these superantigens. Table 5.2 summarises the sites.

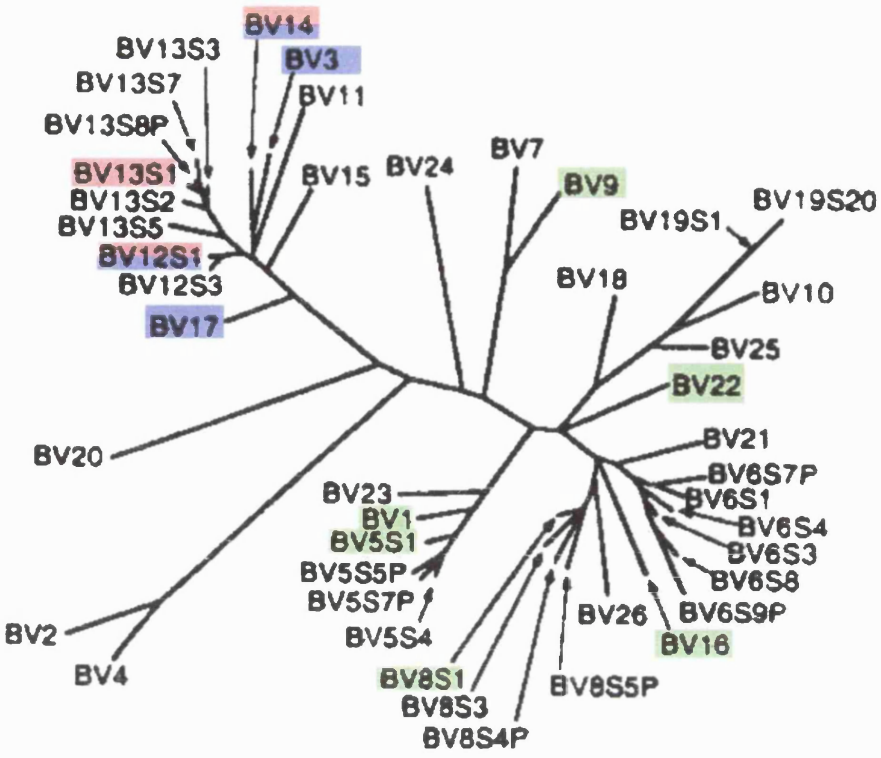


Fig. 5.22. Genetic relatedness of *TRBV* genes. Dendrogram illustrating the nucleotide sequence genetic relatedness of human *TRBV* genes, adapted from Arden *et al* 1995. The main *TRBV* targets of superantigens SPEA, SEB and SEA are highlighted in red, blue and green respectively.

Table 5.2. Sites of SPEA and SEB interaction with mTCRVβ8.2 (Sundberg, Li *et al.* 2002). Interactions are van der Waals contacts unless otherwise indicated as being hydrogen bonds with Vβ main or side chain atoms.

	SPEA – TCR		SEB - TCR	
CDR1	N28	H side	No contacts	
CDR2/FR3	Y50		H47	
	A52		Y50	
	G53	H main	A52	
	S54		G53	H main chain
	T55	H main and side	S54	
	E56	H side	T55	H main chain
	K57	H side	E56	
			K57	H side chain
HV4/FR3	K66		K66	
	A67	H main	A67	
	P70	H main	P70	H main
	S71		S71	
CDR3	No Contacts		No Contacts	

Comparison of TRBV amino acid sequences (appendix 1) using the data presented here, which precisely define the Vβ targets of SPEA and SEB, and the identified sites of TCR binding tabulated above, makes it possible to identify the Vβ sequence and structure features which determine the response to these superantigens. The first notable difference between SEB and SPEA binding sites on TCRVβ is a hydrogen bond formed by the side chain of N28 in CDR1 and E94 of SPEA, an interaction which can not take place with SEB because of its longer disulphide loop (19 *vs* 10 amino acids). The majority of human TCRs have a glycine at this position and hydrogen bond with SPEA would not form (Berman, Westbrook *et al.* 2000). Vβ12, 13 and 14 have N28 while Vβ3 uniquely has aspartic acid at this position which does allow hydrogen bond formation with E94 of SPEA (Berman, Westbrook *et al.* 2000).

Several features of the mVβ8.2 CDR2 have been identified as important in the conformation of this region; serine at position 49 for example and arginine at position 69 (Bentley and Mariuzza 1996). There is a striking match between the Vβs responsive to SPEA and SEB and the presence of S49 and R69 in CDR2 (Appendix 1). A second important difference is the number and nature of bonds between SPEA and SEB and sites in the TCR CDR2. The SPEA interaction with CDR2, involves more hydrogen bonds than does the SEB interaction. Furthermore in the case of SPEA, these hydrogen bonds involve more side chain interactions, are thus more susceptible to amino acid sequence changes. (Sundberg, Li *et al.* 2002). This difference may explain SPEA's targeting of only some of TCRVβs sharing the mVβ8.2 CDR2 structure. Only mVβ8.2, hVβ12, 13 and 14 have N28 in CDR1, the CDR2 features of S49, R69, and have a T55, [DEN]56, K57 sequence in CDR2

allowing Vβ side chain atom hydrogen bonds with SPEA. The next most similar other Vβ, in terms of CDR2 sequence, is Vβ3 in which lysine is found at position 55. For example Vβ17 has N28 and the CDR2 features mVβ8.2 but F55 and Q56 restrict hydrogen bond formation in this region (Berman, Westbrook *et al.* 2000).

In contrast with SPEA, data presented here and in the published literature show relatively promiscuous activation of Vβ types by SEB (Marrack and Kappler 1990; Micusan and Thibodeau 1993). From the above analysis it would appear that the main Vβ feature required for SEB interaction is the CDR2 structure found in mVβ8.2. The Vβs I have found to be targeted at higher SEB concentrations share K57 in CDR2, which is in keeping with this being the site of the only side chain atom hydrogen bond made by SEB. It may be that in Vβs such as Vβ5, 22 and 1 the presence of K57 allows some interaction of SEB with the TCR even though the ideal CDR2 strucure is not present. The failure of Vβ13 to respond to SEB is a striking exception and it is only possible to conjecture that differences in CDR2 away from sites of direct SEB interaction might be responsible for conformational changes which prevent SEB binding.

The relationship demonstrated here between superantigen concentration and the repertoire of responding Vβs makes sense of the differences found in Vβ response depending on HLA class II. Just as there is a spreading out of the Vβ targets as superantigen concentration rises, at any specific concentration the range of Vβs responding will be wider if the class II involved in presentation shows more superantigen binding; *DQA1*01* in the case of SPEA and *DR0401* in the case of SEA. Consequently, for example, at a given concentration of SPEA the percentage of the

total T cell repertoire made up by one of the main Vβ targets, Vβ12 or Vβ14 for SPEA, may be lower following stimulation in the presence of *DQA1*01* because expansions of Vβ13 and 3 are also taking place.

5.5 Conclusions

The fundamental pathology which is considered to underlie superantigen mediated diseases is excessive T cell activation. A relationship between cytokine levels including TNFα and outcome in toxic shock has been observed (Miethke, Wahl *et al.* 1992; Miethke, Wahl *et al.* 1993). The data presented here demonstrate that just as interspecies and interisotype differences in MHC class II binding of bacterial superantigens are associated with differences in T cell response, the allelic differences in superantigen - HLA class II binding, identified in chapters 3 and 4, also result in major differences in the magnitude of the T cell response. The importance of this is, evidently, that allelic differences in class II differentiate individuals and therefore provide a mechanism for inter individual variation in the response to a superantigen.

The studies of Vβ repertoire changes associated with superantigen stimulation described in this chapter have revealed several new Vβ targets of the superantigens studied; the Vβ13.1 response to SPEA for example. My finding that the Vβ specific response to a superantigen is determined by both concentration of superantigen and the class II involved in presentation is entirely novel. This observation provides an explanation for the fact that Vβ targets of a superantigen reported in the literature often differ. It also gives an insight into the role of Vβ structure and sequence features in determining the Vβ targets of a superantigen. The following chapter will discuss

how T cells targeted by superantigens may be important in inducing autoimmunity. The data in this chapter therefore suggest a link between HLA class II and susceptibility to superantigen triggered autoimmunity.

CHAPTER 6

Superantigen induced T cell autoreactivity

6.1 Introduction

Exposure to superantigens has been proposed as a possible trigger for autoimmunity (Wucherpfennig 2001). The theory behind this suggestion can be summarised as follows. Non-specific T cell expansion triggered by superantigen exposure might include self-reactive T cell clones, which may be present in the periphery of healthy individuals in small numbers (Burns, Rosenzweig *et al.* 1983). Expansion of such T cells, possibly to a critical mass, or possibly in conjunction with tissue damage, could initiate a targeted immune response against self. The paucity of experimental and clinical data to support this contention has been discussed in chapter 1.

The work described in this chapter was instigated by the unexpected detection of Vβ11 T cells in HLA-DQ transgenic mice surviving a near lethal dose of SPEA. Since these animals delete Vβ5, 11 and 12 during thymic development as a result of *mtv*8 and *mtv*9 expression in the context of the HLA-DQ transgene, the Vβ11 cells detected represent an autoreactive population of T cells expanded by exposure to a superantigen.

Human equivalents of *mtv* do not exist and to extend these observations to human immunology it was necessary to design *in vitro* experiments aimed at detecting changes in T cell recognition of self antigen following superantigen exposure.

Experimental systems for the study of T cell autoimmunity are probably better established for multiple sclerosis (MS) than any other autoimmune disease (Meinl and Hohlfeld 2002). MS is a chronic inflammatory autoimmune disease of the central nervous system in which the underlying pathology is believed to be the development of T cells reactive against self myelin. The specificities of such autoreactive T cells vary between individuals and during the course of disease but target epitopes of myelin components including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) and proteolipid protein (PLP) (Sun, Olsson *et al.* 1991; Kerlero de Rosbo, Milo *et al.* 1993). T cells recognising epitopes of myelin may also be derived from blood of healthy donors (Ota, Matsui *et al.* 1990).

By studying T cell responses to peptide fragments of these proteins in PBMCs from healthy donors and in PBMCs stimulated with SPEA *in vitro*, expansion of T cells responsive to an epitope of PLP was detected. The specificity of this observation was confirmed by demonstration that the superantigen SMEZ did not produce this effect on any of the myelin epitopes tested. However, *in vivo* exposure of DQ transgenic mice to SPEA did not produce a similar expansion of T cells reactive against any of the murine MBP epitopes tested. The implications of these observations for our understanding of how superantigens may unmask autoimmunity, and how these observations may be extended, are discussed.

6.2 Methods

6.2.1 HLA-DQ transgenic mouse lines

C57/BL6 DQ8 (Boyton, Lohmann *et al.* 1998) transgenic mice carrying the genomic constructs for *DQA1*0301* and *DQB1*0302* and *HLA-DQA1*0102* and *HLA-DQB1*0602* respectively on a mouse class II knock-out background (Cosgrove, Gray *et al.* 1991), were generated by Dr. D. Altmann (Human Disease Immunogenetics Group, Imperial College London). C57/BL6 mice were used as controls. Groups of transgenic and control mice were age (8-14 weeks) and sex matched.

6.2.2 *In vivo* administration of SPEA

In experiments aimed at producing, near lethal exposure to SPEA, HLA-DQ transgenic mice received 20mg of D-galactosamine (D-gal, Sigma, UK) or sterile saline intraperitoneally (i.p.). Ten minutes later, they received a second i.p. inoculation of 20μg SPEA in 0.2ml of saline. Survival was monitored at regular intervals until 8 hours and after 24 hours survivors were killed by cervical dislocation.

In experiments aimed at determining the effect of sub-lethal SPEA exposure on T cell recognition of self antigen, mice received two i.p. doses of 20μg SPEA without D-gal 24 hours apart and were killed by cervical dislocation at 48 hours.

6.2.3 *in vitro* stimulation of PBMCs with SPEA

In experiments aimed at determining the responses of T cells following stimulation by SPEA, $1x10^6$ PBMCs were stimulated in flat bottomed 24 well plates with SPEA at a range of concentrations from 1 to 100ng/ml. After 48 hours cells were washed

and resuspended in fresh medium at $1x10^6$ cells per well and divided after 48 hours and again after 96 hours. At this time only scanty blasts were visible. Cells were then washed and resuspended at $1x10^6$ cells/ml for the peptide stimulation assay.

6.2.4 Human PBMC response to myelin related peptides

A panel of human myelin peptide epitopes derived from MBP, MOG and PLP (MRC Proteomics Group, Imperial College, London), and previously documented as being presented by a wide range of HLA types, was used. T cell proliferation responses to these peptides were sought in fresh PBMCs and in T cell lines expanded by SPEA. Cells were resuspended in HL-1 serum free medium (supplemented with glutamine, penicillin and streptomycin) at 1 $x10^5$ cells/well at a final concentration of 50μg/ml. Plates were tritiated and harvested as described in chapter 2.

6.2.5 Mouse splenocyte response to MBP peptides

A peptide panel of murine MBP 20mers, overlapping by 10 amino acids, was used. The peptides were synthesized by Prof. N. Groome, Oxford Brookes University, UK. Splenocytes were prepared from naïve or SPEA treated mice as described in chapter 2. T cell responses were analyzed using spleen cell cultures resuspended in HL-1 serum free medium (supplemented with glutamine, 2-betamercaptoethanol, penicillin and streptomycin) at $3x10^5$ cells/well in flat-bottom 96 well tissue culture plates. MBP peptides were added to culture wells in triplicate at a final concentration of 50μg/ml. Plates were tritiated and harvested as described in chapter 2.

6.3 Results

6.3.1 *In vivo* Vβ repertoire changes following SPEA exposure in DQ transgenic mice.

The increased susceptibility of HLA-DQ transgenic mice to the toxic effects of SPEA has been demonstrated previously. These animals, by virtue of their transgene, develop markedly more severe manifestations of infection by SPEA producing strains of *S. pyogenes* and frequently die following exposure to recombinant toxin. During infection by *spea+ S. pyogenes* a marked reduction in the number of Vβ8.1 T cells has been noted (Sriskandan, Unnikrishnan *et al.* 2001). Comparison was made of the Vβ repertoire in naïve HLA-DQ transgenic mice with C57/BL6 controls, and of Vβ repertoire changes in these mouse strains after *in vivo* exposure to SPEA. Groups of six D-gal sensitized mice received SPEA. As expected 50% mortality was observed in the DQ transgenic group compared with 100% survival in the C57/BL6 group (data not shown). The Vβ repertoires of the three surviving DQ transgenic mice and three of the control mice were analysed by FACS at 24 hours (Fig. 6.1). The first observation was that the Vβ repertoire of naïve DQ transgenic mice contains very marked deletions of Vβ5 and Vβ11 positive T cells. This was expected because Vβ5 and Vβ11 are two of the Vβs targeted by *mtv*8 and *mtv9*. As previously discussed, mice carrying these endogenous retroviral superantigens delete the target Vβs from their T cell repertoire in the presence of H-2E expression. C57/BL6 mice, because they no not express H2-E, do not delete these Vβs. It has previously been shown that HLA-DQ is able to substitute for H-2E in this regard (Zhou, Smart *et al.* 1992).

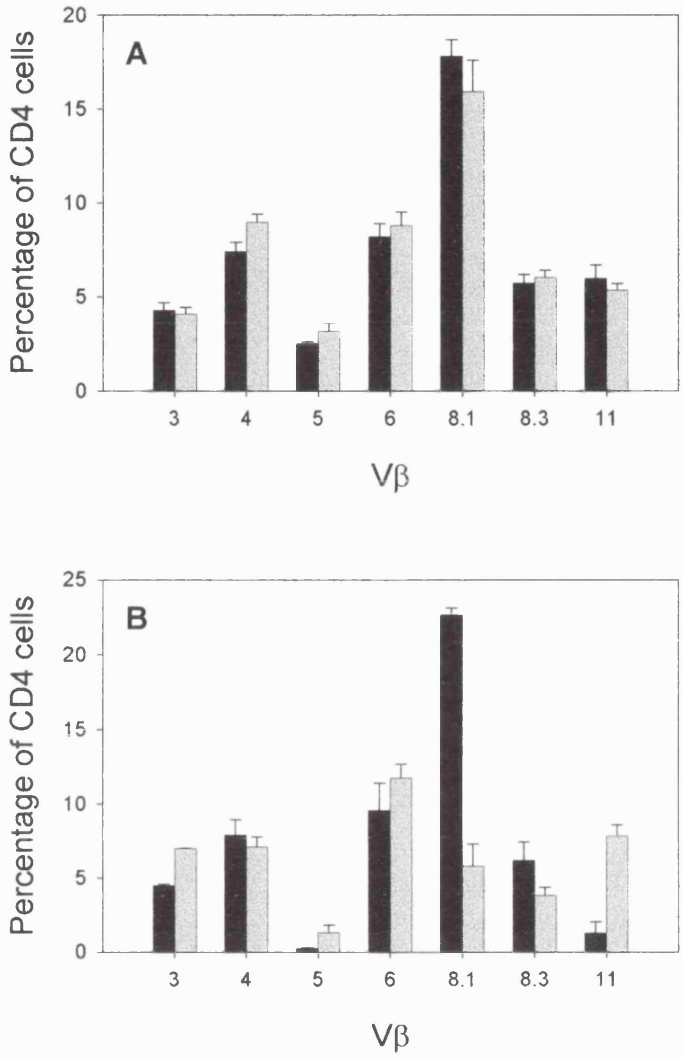


Fig. 6.1. Influence of *in vivo* exposure to SPEA on Vβ repertoire of mouse splenocytes. A) C57/BL6 mice and **B)** HLA-DQ8 AbO transgenic mice splenocytes. Vβ repertoire of splenocytes from naïve mice (■) mice surviving at 24 hours after D-gal sensitization and SPEA 20μg i.p. (□), n=3 in each group, means +/- 1SD are shown.

The marked reduction in Vβ8.1 T cells observed in the HLA-DQ transgenic mice following SPEA exposure is in keeping with this Vβ being targeted by SPEA and the Vβ changes which have been observed in HLA-DQ transgenic mice following infection by SPEA producing strains of *S. pyogenes*. Unexpectedly, the Vβ repertoire of HLA-DQ transgenic mice after exposure to SPEA contained increased proportions of Vβ11, and to a lesser extent Vβ5, CD4 cells. FACS analysis clearly demonstrated that the Vβ11 positive CD4 cells represented a distinct population that was not present in naïve mice (Fig. 6.2).

6.3.2 Effect of *in vitro* exposure to superantigen on human PBMC responses to self antigen

In order to determine whether superantigens might be able to expand populations of autoreactive T cells in man, experiments were designed to seek an effect of *in vitro* exposure to superantigens on T cell recognition of a panel of peptides derived from antigens implicated in the aetiology of MS. T cell responses were sought to ten peptide fragments of MBP, PLP and MOG in three healthy donors. In fresh PBMCs from these donors a response to MOG 76-100 was detected in one donor, PLP 30-49 in another and MOG 34-46, MBP 13-32, MOG 76-100 and PLP 185-206 in the third (Fig. 6.3). Following stimulation with SPEA at a range of concentrations from 1ng/ml to 100ng/ml T cell responses to this same panel of epitopes had become dramatically focused. In all three donors a stimulation index of five or more was found in response to PLP30-49. Responses to all the other peptides tested were below 2. Interestingly PPD responses were also lost (Fig 6.4a). In order to determine whether this

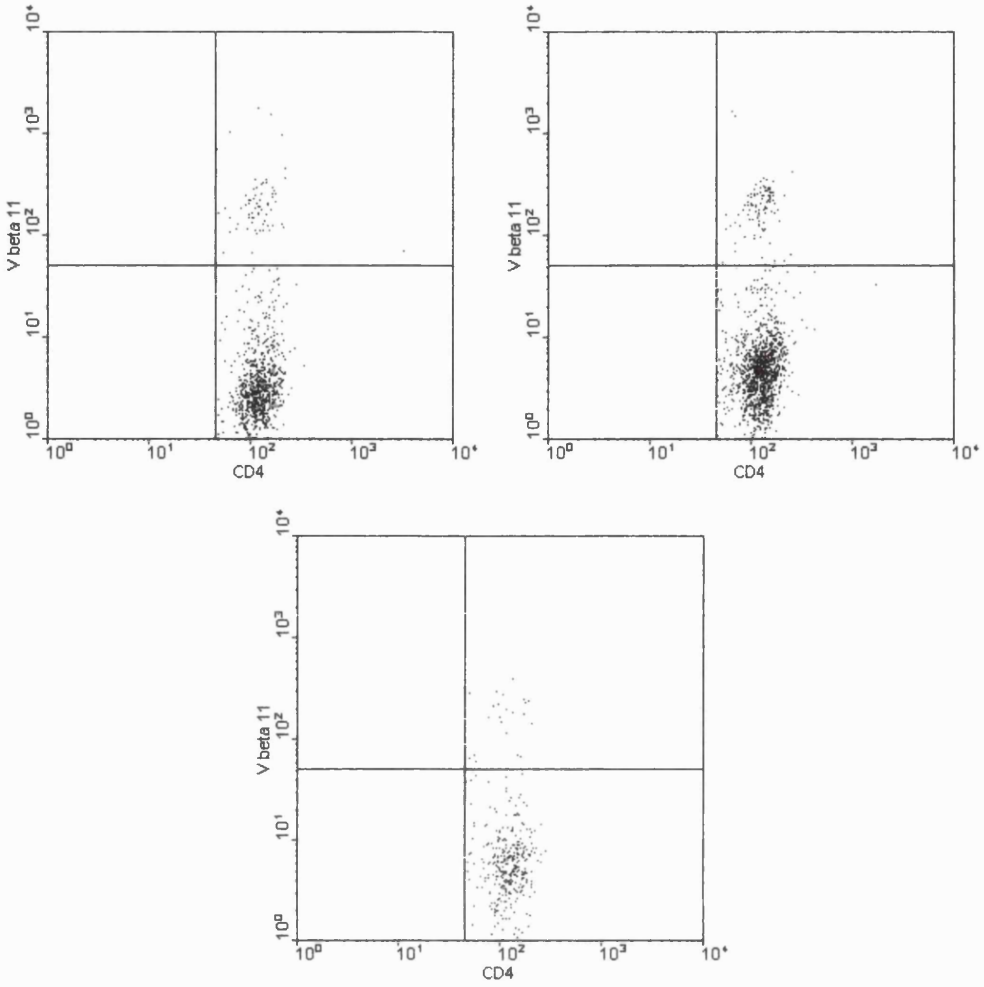


Fig. 6.2. FACS plots of Vβ11 lymphocytes in DQ3 mouse splenocytes following *in vivo* exposure to SPEA. The results from three mice are shown.

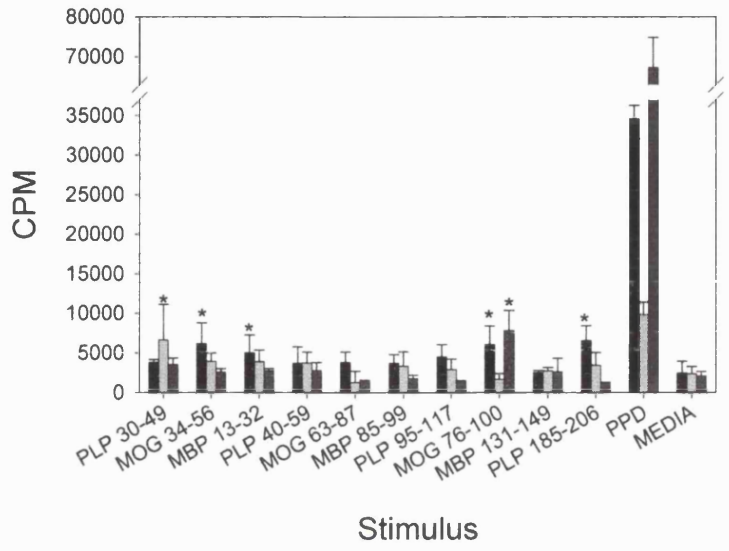


Fig. 6.3. Base-line proliferation of donor PBMCs in response to MS peptides. Mean (n=3) responses from three individuals are shown +/- 1SD. Responses >2 fold higher than in the presence of media alone are marked *.

effect on T cells responsive to PLP30-49 might be a general effect of superantigen stimulation, the experiment was repeated using SMEZ in place of SPEA. In T cells stimulated by SMEZ, no response to PLP30-49, or any of the other peptides tested, was found (Fig. 6.4b).

The donors used in these experiments were not selected to be any particular HLA type, one was HLA-DQ2,8, and two were HLA-DQ2,5. To explore the possibility that differences in HLA-DQ binding and presentation SPEA might influence the selection of T cells recognising PLP30-49 the experiment was repeated in two donors, one DQ5,6 (*DQA1*01* homozygous) and one DQ8,8 (*DQA1*03* homozygous). The same effect of SPEA stimulation resulting in a population of PLP30-49 responsive T cells was observed in both these donors (Fig. 6.5).

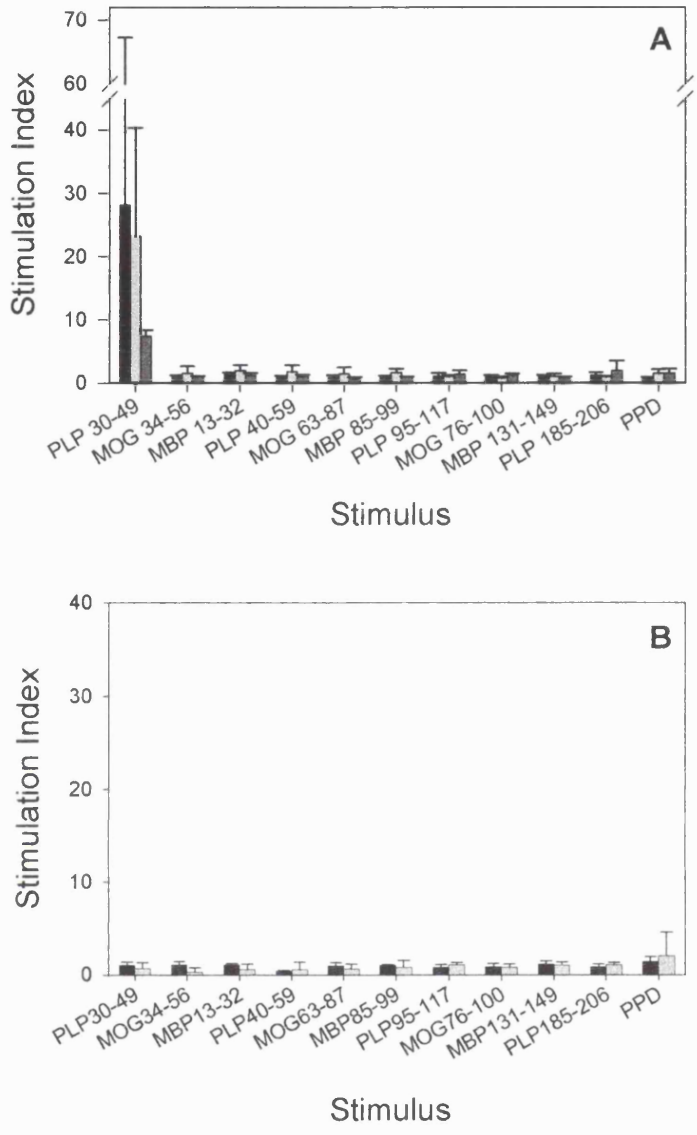


Fig. 6.4. Influence of superantigen exposure on T cell responses to MS peptide panel. A) Proliferation of T cells expanded by stimulation with SPEA at 100 (■), 10 (▭), 1 (▭) ng/ml. **B)** Proliferation of T cells expanded by stimulation with SMEZ at 1000 (■) and 100 (▭) pg/ml. Each bar is a mean of the same three donors +/- 1sd.

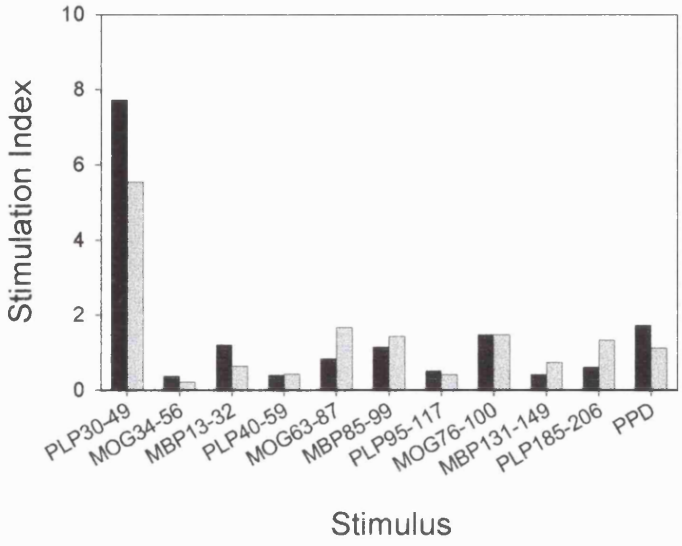


Fig. 6.5. Influence of SPEA exposure on T cell responses to MS peptide panel; influence of HLA-DQ. T cells from two donors, one *HLA-DQA1*01* homozygous (■), one *HLA-DQ A1*03* homozygous (□) after stimulation with SPEA at 500ng/ml.

6.3.3 Vβ repertoire of a PLP30-49 derived T cell line

A possible explanation for finding that T cells expanded by SPEA recognise the self antigen PLP30-49 is that TCRs with this specificity are particularly linked to one of the Vβs targeted by SPEA. To test this possibility a T cell line was derived from the single donor shown in Fig. 6.1, in whom a base-line response to PLP30-49 had been identified. The line was established by three rounds of stimulation with PLP30-49 at weekly intervals. The Vβ repertoire of the line, directed at Vβ types targeted by SPEA, was determined at four weeks and compared with the base-line Vβ repertoire. No increase in the proportions of any of the Vβs previously identified as responding to SPEA was detected. In fact the proportion of each Vβ analysed fell compared with baseline, indicating skewing of the Vβ response in PLP30-49 responsive T cells away from those Vβs targeted by SPEA (Fig. 6.6). It is particularly striking that Vβ12, 13.1 and 14 are markedly absent from the T cell line suggesting that these Vβs are excluded from TCRs with specificity for PLP30-49.

6.3.4 Murine response to self antigen following *in vivo* exposure to SPEA.

To determine whether SPEA exposure *in vivo* can unmask responses to peptide self antigen the response to murine splenocytes to a panel of murine MBP peptides was determined in naïve mice and in mice exposed to sub-lethal doses of SPEA. Although in each case a positive control response to concavalin A was seen, no peptide responses were found in either naïve or SPEA treated mice (data not shown).

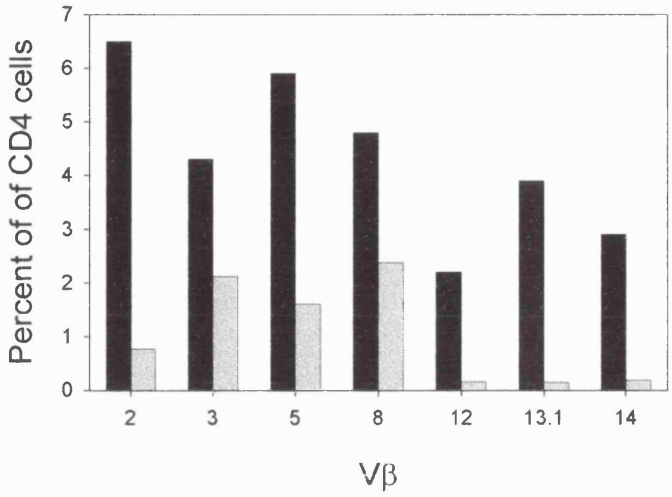


Fig. 6.6 Vβ repertoire of PLP30-49 derived T cell line. The percentage of CD4 positive cells in fresh PBMCs (■) and a T cell line derived by three rounds of stimulation with PLP30-49 (□) and in the same donor are shown.

6.4 Discussion

Experimental evidence supporting the contention that superantigen exposure can trigger autoimmunity is scarce (Wucherpfennig 2001). No reports exist of an association between the best defined syndromes of superantigen exposure, the toxic shock syndromes, and subsequent development of autoimmunity in survivors. Nevertheless, as was discussed in Chapter 1, a number of sequelae of *S. pyogenes* infection do have an autoimmune basis including rheumatic fever, glomerulonephritis and Sydenham's chorea. Additionally, superantigens are implicated in the aetiology of several diseases characterised by autoimmunity including Kawasaki's disease and psoriasis.

The work described in this chapter contains several tantalising pieces of evidence that superantigens can trigger mechanisms of autoimmunity. Mice transgenic for HLA-DQ on a mouse class II negative background, in keeping with the known expression of *mtv*8 and 9 in these animals, were found to delete Vβ11 T cells. In naïve HLA-DQ transgenics, less than 1% of CD4 cells were Vβ11 compared with 6% in the C57/BL6 controls. The detection of these cells might be attributable to a technical failure of flow cytometry to detect the absence of a population of cells. However the presence of Vβ11 cells following near lethal exposure to SPEA suggests that complete deletion of Vβ11 in these animals has not taken place. Partial deletion of *mtv* targeted Vβs in mice carrying transgenes HLA class II transgenes has been described previously (Altmann, Takacs *et al.* 1993). This does not diminish the importance of the observation. Rather it is likely that leakage of a few Vβ11 T cells in to the peripheral immune system of these mice is no different from the leakage which allows

autoreactive T cells to be cloned from peripheral sites under normal circumstances (Burns, Rosenzweig *et al.* 1983). The murine Vβ target of SPEA is Vβ8.2. While homology exists between other murine Vβs and Vβ8.2, Vβ11 has never been implicated as a target of SPEA and shares none of the CDR2 sequence characteristics established in chapter 5 which might suggest that it could be a secondary target for SPEA.

The finding that T cells expanded by *in vitro* exposure to SPEA are able to mount peptide specific responses to the same auto antigen, PLP30-49, in all donors tested, irrespective of whether a response to this peptide was detectable in fresh PBMCs, indicates that T cells with this specificity are present in the peripheral immune system. Even so the Vβ repertoire of T cells expanded by stimulation with PLP30-49 was not found to be skewed towards any of the Vβs targeted by SPEA. Under the conditions of SPEA stimulation used it is worth emphasising that the results in chapter 5 indicate 100% of the CD4 repertoire is accounted for by Vβ3, 12, 14, and 13.1. This finding can not be interpreted therefore as indicating that TCRs specific for PLP30-49 are skewed towards another SPEA targeted Vβ not accounted for in the panel. The observation that TCRs of Vβ12, 13.1 and 14 are notably absent from the Vβ repertoire of the T cell line implies that these Vβs are excluded from TCRs with specificity for this peptide.

Taken together these results can not be explained by a model of superantigen triggered autoimmunity in which superantigen expansion of autoreactive T cells is the fundamental process. Indeed the reverse situation seems to be the case. Another process attributable to superantigenicity must be responsible; superantigen induced T

cell anergy or deletion for example. It is possible that anergy of regulatory T cells might permit populations of autoreactive T cells, particularly if their TCR specificity is based on Vβs not targeted in the superantigen response, to become established.

To further explore these possibilities it would be important to characterise in detail the TCRs of PLP30-49 specific clones derived from PBMCs in donors with base-line reactivity to this peptide and derived from SPEA expanded T cells. It would also be useful to phenotype the T cells which, following SPEA stimulation, mount a response to PLP30-49. From a clinical perspective it would be very interesting to look at T cell responses to self antigen, particularly antigens derived from tissues targeted in the immunological sequelae of *S. pyogenes* infection, in patients during and after streptococcal infections. Matching of T cell responses and superantigen production by the patients own streptococcal isolates would then be possible. Since it is likely that selection of autoreactive T cells may occur much more commonly than clinical auto immune disease it might be feasible to prospectively characterise T cell reactivity to self antigens in a cohort of individuals before and after superficial streptococcal infections.

The single pilot experiment described here looking for an influence of HLA-DQ on SPEA selection of autoreactive T cells produced a negative result. Nevertheless, epidemiological data strongly suggest that HLA class II associated differences in superantigen selection of autoreactive T cells do exist. While an epidemiological link between HLA class II and toxic shock syndrome is limited to the single recent study of Kotb *et al.*, associations have been identified for Kawasaki's disease and the immunological sequelae of *S. pyogenes* infection (Ayoub, Barrett *et al.* 1986;

Guilherme, Weidebach *et al.* 1991; Barron and Silverman 1992; Fildes, Burns *et al.* 1992; Olmez, Turgay *et al.* 1993; Visentainer, Pereira *et al.* 2000). This suggests that further study of the novel observation reported in this chapter would be worthwhile.

6.5 Conclusions

The work described in this chapter makes several novel, albeit preliminary, observations supporting the concept that superantigens can trigger specific auto immunity. SPEA is shown to be able to reveal populations of autoreactive T cells in two experimental systems. The results suggest that superantigen expansion of self-reactive T cells can not alone account superantigen induced autoimmunity. T cells which escape superantigen deletion or anergy may responsible. The autoimmune sequelae of *S. pyogenes* infection are commonly explained by molecular mimicry. These data indicate that the role of superantigens in post-streptococcal autoimmunity should now be studied in detail.

CHAPTER 7

Final discussion and future work

This project was stimulated by the question of why individuals differ in susceptibility to superantigen mediated shock. Specifically, the project was designed to address the possibility that HLA class II polymorphisms could influence superantigenicity since the interaction with class II is of such central importance in the response to superantigens. During the three years over which this work was performed Kotb *et al* published data confirming the existence of an HLA class II haplotype association with the severity of *S. pyogenes* infection (Kotb, Norrby-Teglund *et al.* 2002). Their study was primarily epidemiological but they also used partially purified bacterial culture supernatants to extend their observations *in vitro*. As I have already discussed, because clinical strains of *S. pyogenes* will invariably carry genes for multiple superantigens, and because multiple strains of *S. pyogenes* were present in the population they studied, neither the *in vitro* or *in vivo* data presented in Kotb's study addresses the underlying mechanism of the association they observed. By making a detailed study of the binding to HLA class II of three bacterial superantigens; SPEA, SEA and SEB, I have been able to demonstrate that HLA class II polymorphisms determine the strength of superantigen binding. While inter-isotype differences in HLA class II binding of superantigens have been demonstrated by Kotb's group and others (Imanishi, Igarashi *et al.* 1992; Norrby-Teglund, Nepom *et al.* 2002), I have

demonstrated differences at a sub-isotype level. This is of crucial importance since it provides a mechanism for inter-individual differences. The differences in binding of superantigens were associated with differences both in the magnitude and the quality of the T cell response. I have speculated that the apparent contrast between Kotb's finding that *HLA-DQA1*0102/DQB1*0602* is associated with protection from streptococcal toxic shock and my observation that *HLA-DQA1*01* presentation of SPEA elicits a more powerful T cell response, may be due to the presence of multiple superantigens, in particular more potent superantigens such as SMEZ, in the purified bacterial culture supernatants used in Kotb's study. One direct extension of my work would be to analyse the interaction of SMEZ with class II using the approaches I have described in this thesis. This would also serve to determine whether complementary usage of class II by superantigens co-produced by a single organism does indeed occur.

The implications of the findings presented here go beyond susceptibility to superantigen mediated shock. The precise role played by superantigens in the life cycle of bacteria remains unclear. Nevertheless, the existence of such a diversity of superantigens in *S. aureus* and *S. pyogenes* attests to the evolutionary advantage these toxins must confer. Since colonisation and transmission of both *S. aureus*, on the skin, and *S. pyogenes* in the pharynx, occur at superficial sites, it seems likely that superantigens function to prolong carriage, facilitate transmission or prevent generation of host immunity at these sites. HLA associations with susceptibility to individual superantigens may therefore influence duration of *S. pyogenes* carriage in the pharynx and predisposition to recurrent streptococcal pharyngitis. Additionally

superantigens are implicated in enterotoxic food poisoning, culture negative sepsis and progression from superficial streptococcal infection to invasive disease in the absence of toxic shock. HLA class II may therefore be linked to disease susceptibility across the whole spectrum of disease associated with *S. pyogenes* and *S. aureus* infection. HLA class II determined susceptibility to superantigens would be expected to have an impact, not only on individual susceptibility to the effects of a superantigen but also, on the susceptibility of in-bred populations to infection by superantigen producing organisms.

The importance of subtle differences in class II at the sites of superantigen binding demonstrated in chapters 3 and 4 suggests that this may be a site at which potential therapeutic interventions could be targeted. The ELISA I have developed to detect superantigen binding to class II in solution would be a useful tool in screening such potential agents. Further study of peptide inhibitors such as the *DQA1*01(53-73)* peptide studied here is indicated. Monoclonal antibodies raised against regions of the class II molecule involved in superantigen binding might have therapeutic value if they could be shown to have superantigen blocking activity but not block presentation of conventional antigen by class II.

The data presented in Chapter 5 of this thesis first of all confirm the functional importance of differences detected in binding both for SPEA and SEA. The magnitude of the T cell response both in terms of proliferation and cytokine production depends on the class II involved in superantigen presentation. Secondly, the Vβ specific T cell response to superantigens *in vitro* is shown by the data presented here, to be determined by superantigen concentration with the range of Vβs

involved spreading out as superantigen concentration rises. The HLA class II involved in superantigen presentation modulates this effect. HLA class II molecules exhibiting greater binding of an individual superantigen produce a wider Vβ repertoire of response at a lower concentration of superantigen than class II molecules exhibiting lower binding. It would be difficult to design experiments to determine whether this effect occurs *in vivo*. Comparisons using different strains of HLA transgenic mice would be confounded by differences in level and tissue distribution of class II expression. Furthermore superantigen responsive T cells are generally observed to be deleted *in vivo* and the repertoire of murine Vβs responsive to superantigens is generally limited. These findings have several important implications. They may explain discrepancies in reported Vβ repertoire changes both for individual superantigens *in vitro* and clinically in Kawasaki's disease and toxic shock. They may also link HLA class II and superantigen induced autoimmunity.

The role of superantigens in the aetiology of the autoimmune sequelae of *S. pyogenes* infection is unclear but their role in triggering autoimmunity in Kawasaki's disease is well established. The theoretical basis for superantigen induced autoimmunity has been discussed in chapter 6. It is noteworthy that prior to Kotb's observations the only manifestation of streptococcal infection with well documented HLA associations is rheumatic fever (Ayoub, Barrett *et al.* 1986; Guilherme, Weidebach *et al.* 1991; Olmez, Turgay *et al.* 1993; Visentainer, Pereira *et al.* 2000). Numerous associations of other autoimmune diseases with HLA class II exist (Gebe, Swanson *et al.* 2002) and associations with HLA-DQ are particularly puzzling given the relatively poor expression of HLA-DQ by antigen presenting cells and the fact that HLA-DQ

restricted T cells are not commonly isolated in most systems (Douek and Altmann 2000). My observation of an HLA-DQ association with differences in the Vβ specific response to a streptococcal superantigen provides a plausible link between infection and HLA-DQ associated autoimmunity.

The finding of superantigen induced T cell responses to PLP presented in chapter 6 of this thesis, while being preliminary, nevertheless represents a proof-of-principle of how superantigens might link infectious agents with HLA class II predisposition to autoimmune disease. Uncontrolled Vβ expansions in response to superantigens would be expected to encompass many self-reactive T cells. Exposure to superantigens during the course of trivial soft-tissue infections undoubtedly occurs as evidenced by the seroprevalence of superantigen antibodies in the general population (Proft, Moffatt *et al.* 2000). It may be therefore, that transient populations of T cells directed against cardiac muscle occur after streptococcal pharyngitis much more commonly than does clinical rheumatic fever. Investigation of the immunological consequences of superantigen exposure during common and trivial Streptococcal and Staphylococcal infections is warranted.

It is intriguing that *S. aureus* and *S. pyogenes*, both being species specific pathogens of man, are distinct in their use of superantigens and have each evolved multiple superantigen toxins. My findings suggest that HLA class II diversity could be responsible for driving the generation of superantigen diversity in these organisms. Numerous examples of human genetic diversity having arisen in response to infection are established (Hill 1998) but this may be an example of human genetic

heterogeneity driving the evolution of diversity among some of Man's most dangerous pathogens.

CHAPTER 8

References

1. Abe, J., J. Forrester, *et al.* (1991). "Selective stimulation of human T cells with streptococcal erythrogenic toxins A and B." Journal of Immunology **146**(11): 3747-50.

2. Abe, J., B. L. Kotzin, *et al.* (1993). "Characterization of T cell repertoire changes in acute Kawasaki disease." Journal of Experimental Medicine **177**(3): 791-6.

3. Abe, J. and T. Takeda (1997). Characerization of a superantigen produced by Yersinia pseudotuberculosis. Superantigens: Molecular Biology, Immunology and Relevance to Human Disease. D. Y. Leung, B. T. Huber and P. M. Schlievert. New York, Marcel Dekker: 369.

4. Abe, R., J. J. Ryan, *et al.* (1987). "Clonal analysis of the Mls system. A reappraisal of polymorphism and allelism among Mlsa, Mlsc, and Mlsd." Journal of Experimental Medicine **165**(4): 1113-29.

5. Acha-Orbea, H., A. N. Shakhov, *et al.* (1991). "Clonal deletion of V beta 14-bearing T cells in mice transgenic for mammary tumour virus." Nature **350**(6315): 207-11.

6. Alber, G., D. K. Hammer, *et al.* (1990). "Relationship between enterotoxic- and T lymphocyte-stimulating activity of staphylococcal enterotoxin B." Journal of Immunology **144**(12): 4501-6.

7. al-Daccak, R., K. Mehindate, *et al.* (1994). "Signalling via MHC class II molecules selectively induces IL-1 beta over IL-1 receptor antagonist gene expression." Biochemical and Biophysical Research Communications **201**(2): 855-60.

8. Altekruse, S. F., M. L. Cohen, *et al.* (1997). "Emerging foodborne diseases." Emerging Infectious Diseases **3**(3): 285-93.

9. Altmann, D. M., K. Takacs, *et al.* (1993). "Mouse mammary tumor virus-mediated T-cell receptor negative selection in HLA-DRA transgenic mice." Human Immunology **37**(3): 149-56.

10. Andersen, L. C., J. S. Beaty, *et al.* (1991). "Allelic polymorphism in transcriptional regulatory regions of HLA-DQB genes." Journal of Experimental Medicine **173**(1): 181-92.

11. Andrews, M. M., E. M. Parent, *et al.* (2001). "Recurrent nonmenstrual toxic shock syndrome: clinical manifestations, diagnosis, and treatment." Clinical Infectious Diseases **32**(10): 1470-9.

12. Arad, G., R. Levy, *et al.* (2000). "Superantigen antagonist protects against lethal shock and defines a new domain for T-cell activation." Nature Medicine **6**(4): 414-21.

13. Arden, B., S. P. Clark, *et al.* (1995). "Human T-cell receptor variable gene segment families." Immunogenetics **42**(6): 455-500.

14. Ayoub, E. M., D. J. Barrett, *et al.* (1986). "Association of class II human histocompatibility leukocyte antigens with rheumatic fever." Journal of Clinical Investigation **77**(6): 2019-26.

15. Baker, M., D. M. Gutman, *et al.* (2001). "Structural features of a zinc binding site in the superantigen strepococcal pyrogenic exotoxin A (SpeA1): Implications for MHC class II recognition." Protein Science **10**(6): 1268-1273.

16. Balaban, N. and A. Rasooly (2000). "Staphylococcal enterotoxins." International Journal of Food Microbiology **61**(1): 1-10.

17. Banks, D. J., S. B. Beres, *et al.* (2002). "The fundamental contribution of phages to GAS evolution, genome diversification and strain emergence." Trends In Microbiology **10**(11): 515-21.

18. Bannan, J., K. Visvanathan, *et al.* (1999). "Structure and function of streptococcal and staphylococcal superantigens in septic shock." Infectious Disease Clinics of North America **13**(2): 387-96, ix.

19. Barron, K. S., S. T. Shulman, *et al.* (1999). "Report of the National Institutes of Health Workshop on Kawasaki Disease." Journal of Rheumatology **26**(1): 170-90.

20. Barron, K. S. and E. D. Silverman (1992). "MHC class II alleles in Kawasaki syndrome - lack of consistent correlation with disease or cardiac involvement." Journal of Rheumatology **19**: 1790-1793.

21. Barsumian, E. L., P. M. Schlievert, *et al.* (1978). "Nonspecific and specific immunological mitogenicity by group A streptococcal pyrogenic exotoxins." Infection and Immunity **22**(3): 681-8.

22. Baschieri, S., R. K. Lees, *et al.* (1993). "Clonal anergy to staphylococcal enterotoxin B in vivo: selective effects on T cell subsets and lymphokines." European Journal of Immunology **23**(10): 2661-6.

23. Basma, H., A. Norrby-Teglund, *et al.* (1999). "Risk factors in the pathogenesis of invasive group A streptococcal infections: role of protective humoral immunity." Infection and Immunity **67**(4): 1871-7.

24. Begovich, A. B., W. Klitz, *et al.* (2000). HLA-DQ haplotypes in 15 different populations. Major Histocompatibility Complex - evolution, structure and function. M. Kasahara. Tokyo, Springer-Verlag**:** 412-426.

25. Bentley, G. A. and R. A. Mariuzza (1996). "The structure of the T cell antigen receptor." Annual Review of Immunology **14**: 563-90.

26. Beres, S. B., G. L. Sylva, *et al.* (2002). "Genome sequence of a serotype M3 strain of group A Streptococcus: phage-encoded toxins, the high-virulence phenotype, and clone emergence." Proceedings of The National Academy of Sciences of the United States of America **99**(15): 10078-83.

27. Berman, H. M., J. Westbrook, *et al.* (2000). "The Protein Data Bank." Nucleic Acids Research **28**: 235-242.

28. Bontrop, R. E., G. M. Schreuder, *et al.* (1986). "Molecular, serologic, and functional evidence for an apparent HLA-DR triplet." Journal of Immunolgy **137**(1): 211-6.

29. Boyton, R. J., T. Lohmann, *et al.* (1998). "Glutamic acid decarboxylase T lymphocyte responses associated with susceptibility or resistance to type I diabetes: analysis in disease discordant human twins, non-obese diabetic mice and HLA-DQ transgenic mice." International Immunology **10**(12): 1765-76.

30. Braun, M. A., D. Gerlach, *et al.* (1993). "Stimulation of human T cells by streptococcal superantigen erythrogenic toxins (scarlet fever toxins)." Journal of Immunology **150**(6): 2457-66.

31. Brocke, S., A. Gaur, *et al.* (1993). "Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen." Nature **365**(6447): 642-4.

32. Bunikowski, R., M. E. Mielke, *et al.* (2000). "Evidence for a disease-promoting effect of Staphylococcus aureus-derived exotoxins in atopic dermatitis." Journal of Allergy and Clinical Immunology **105**(4): 814-9.

33. Burns, J., A. Rosenzweig, *et al.* (1983). "Isolation of myelin basic protein-reactive T-cell lines from normal human blood." Cellular Immunology **81**(2): 435-40.

34. Cartwright, K., M. Logan, *et al.* (1995). "A cluster of cases of streptococcal necrotizing fasciitis in Gloucestershire." Epidemiology and Infection **115**(3): 387-97.

35. CCDC (1996). "Streptococcal Toxic-Shock Syndrome (STSS) 1996 Case Definition." http://www.cdc.gov/epo/dphsi/casedef/streptococcalcurrent.htm

36. CCDC (1997). "Toxic Shock Syndrome (TSS) 1997 Case Definition." http://www.cdc.gov/epo/dphsi/casedef/toxicsscurrent.htm

37. Chatellier, S., N. Ihendyane, *et al.* (2000). "Genetic relatedness and superantigen expression in group A streptococcus serotype M1 isolates from

patients with severe and nonsevere invasive diseases." Infection and Immunity **68**(6): 3523-34.

38. Chatila, T. and R. S. Geha (1993). "Signal transduction by microbial superantigens via MHC class II molecules." Immunological Reviews **131**: 43-59.

39. Choi, Y. W., A. Herman, *et al.* (1990). "Residues of the variable region of the T-cell-receptor beta-chain that interact with S. aureus toxin superantigens." Nature **346**(6283): 471-3.

40. Choi, Y. W., B. Kotzin, *et al.* (1989). "Interaction of Staphylococcus aureus toxin superantigens with human T cells." Proceedings of The National Academy of Sciences of the United States of America **86**(22): 8941-5.

41. Chu, F. S., K. Thadhani, *et al.* (1966). "Purification and characterization of staphylococcal enterotoxin A." Biochemistry **5**(10): 3281-9.

42. Cockerill, F. R., 3rd, K. L. MacDonald, *et al.* (1997). "An outbreak of invasive group A streptococcal disease associated with high carriage rates of the invasive clone among school-aged children." Journal of the American Medical Association **277**(1): 38-43.

43. Cole, B. C. and M. M. Griffiths (1993). "Triggering and exacerbation of autoimmune arthritis by the Mycoplasma arthritidis superantigen MAM." Arthritis and Rheumatism **36**(7): 994-1002.

44. Cole, B. C., K. L. Knudtson, *et al.* (1996). "The sequence of the Mycoplasma arthritidis superantigen, MAM: identification of functional domains and comparison with microbial superantigens and plant lectin mitogens." Journal of Experimental Medicine **183**(3): 1105-10.

45. Cone, L. A., D. R. Woodard, *et al.* (1987). "Clinical and bacteriologic observations of a toxic shock-like syndrome due to Streptococcus pyogenes." New England Journal of Medicine **317**(3): 146-9.

46. Cosgrove, D., D. Gray, *et al.* (1991). "Mice lacking MHC class II molecules." Cell **66**(5): 1051-66.

47. Cresswell, P. (1994). "Assembly, transport, and function of MHC class II molecules." Annual Review of Immunology **12**: 259-93.

48. Cunningham, M. W. (2000). "Pathogenesis of group A streptococcal infections." Clinical Microbiology Reviews **13**(3): 470-511.

49. Cunningham, M. W., H. C. Meissner, *et al.* (1999). "Anti-human cardiac myosin autoantibodies in Kawasaki syndrome." Journal of Immunology **163**(2): 1060-5.

50. Curtis, N., R. Zheng, *et al.* (1995). "Evidence for a Superantigen Mediated Process in Kawasaki Disease." Archives of Disease in Childhood **72**: 308-311.

51. Dalwadi, H., B. Wei, *et al.* (2001). "The Crohn's disease-associated bacterial protein I2 is a novel enteric t cell superantigen." Immunity **15**(1): 149-58.

52. Davis, M. M. (1990). "T cell receptor gene diversity and selection." Annual Review of Biochemistry **59**: 475-96.

53. de Azavedo, J. C., T. J. Foster, *et al.* (1985). "Expression of the cloned toxic shock syndrome toxin 1 gene (tst) in vivo with a rabbit uterine model." Infection and Immunity **50**(1): 304-9.

54. Dellabona, P., J. Peccoud, *et al.* (1990). "Superantigens interact with MHC class II molecules outside of the antigen groove." Cell **62**(6): 1115-21.

55. Demers, B., A. E. Simor, *et al.* (1993). "Severe invasive group A streptococcal infections in Ontario, Canada: 1987-1991." Clinical Infectious Diseases **16**(6): 792-800; discussion 801-2.

56. Dessen, A., C. M. Lawrence, *et al.* (1997). "X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II." Immunity 7(4): 473-81.

57. Diener, K., P. Tessier, *et al.* (1998). "Induction of acute inflammation in vivo by staphylococcal superantigens I: Leukocyte recruitment occurs independently of T lymphocytes and major histocompatibility complex Class II molecules." Laboratory Investigation **78**(6): 647-56.

58. Dobrescu, D., B. Ursea, *et al.* (1995). "Enhanced HIV-1 replication in V beta 12 T cells due to human cytomegalovirus in monocytes: evidence for a putative herpesvirus superantigen." Cell **82**: 753-63.

59. Douek, D. C. and D. M. Altmann (2000). "T cell apoptosis and differential HLA class II expression in human thymus." Immunology **99**: 249-256.

60. Duong, T. T., E. D. Silverman, *et al.* (2003). "Superantigenic activity is responsible for induction of coronary arteritis in mice: an animal model of Kawasaki disease." International Immunology **15**(1): 79-89.

61. Earhart, C. A., G. M. Vath, *et al.* (2000). "Structure of streptococcal pyrogenic exotoxin A reveals a novel metal cluster." Protein Science **9**(9): 1847-51.

62. Efstratiou, A. (2000). "Group A streptococci in the 1990s." Journal of Antimicrobial Chemotherapy **45**(Suppl): 3-12.

63. Eriksson, B. K., J. Andersson, *et al.* (1999). "Invasive group A streptococcal infections: T1M1 isolates expressing pyrogenic exotoxins A and B in combination with selective lack of toxin-neutralizing antibodies are associated with increased risk of streptococcal toxic shock syndrome." Journal of Infectious Diseases **180**(2): 410-8.

64. Ettinger, R. A., A. W. Liu, *et al.* (1998). "Exceptional stability of the HLA-DQA1*0102/DQB1*0602 alpha beta protein dimer, the class II MHC molecule associated with protection from insulin-dependent diabetes mellitus." Journal of Immunology **161**(11): 6439-45.

65. Fast, D. J., P. M. Schlievert, *et al.* (1989). "Toxic shock syndrome-associated staphylococcal and streptococcal pyrogenic toxins are potent inducers of tumor necrosis factor production." Infection and Immunity **57**(1): 291-4.

66. Fermand, J. P., A. Chevalier, *et al.* (1986). "Characterization of a monoclonal antibody recognizing a monomorphic determinant of the alpha chain of class II DQ antigens." Scandinavian Journal of Immunology **24**(3): 313-9.

67. Ferretti, J. J., W. M. McShan, *et al.* (2001). "Complete genome sequence of an M1 strain of Streptococcus pyogenes." Proceedings of The National Academy of Sciences of the United States of America **98**(8): 4658-63.

68. Festenstein, H. (1973). "Immunogenetic and biological aspects of in vitro lymphocyte allotransformation (MLR) in the mouse." Transplantation Reviews **15**: 62-88.

69. Fildes, N., J. C. Burns, *et al.* (1992). "The HLA class II region and susceptibility to Kawasaki disease." Tissue Antigens. **39**(2): 99-101.

70. Fleischer, B., A. Necker, *et al.* (1996). "Reactivity of mouse T-cell hybridomas expressing human Vbeta gene segments with staphylococcal and streptococcal superantigens." Infection and Immunity **64**(3): 987-94.

71. Fleischer, B. and H. Schrezenmeier (1988). "T cell stimulation by staphylococcal enterotoxins. Clonally variable response and requirement for major histocompatibility complex class II molecules on accessory or target cells." Journal of Experimental Medicine **167**(5): 1697-707.

72. Florquin, S., Z. Amraoui, *et al.* (1994). "Systemic release and protective role of IL-10 in staphylococcal enterotoxin B-induced shock in mice." Journal of Immunology **153**(6): 2618-23.

73. Folch, G. and M. P. Lefranc (2000). "The human T cell receptor beta variable (TRBV) genes." Experimental and Clinical Immunogenetics **17**(1): 42-54.

74. Fraser, J., V. Arcus, *et al.* (2000). "Superantigens - powerful modifiers of the immune system." Molecular Medicine Today **6**(3): 125-32.

75. Fraser, J. D. (1989). "High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR." Nature **339**(6221): 221-3.

76. Gebe, J. A., E. Swanson, *et al.* (2002). "HLA class II peptide-binding and autoimmunity." Tissue Antigens **59**(2): 78-87.

77. Gerlach, D., B. Fleischer, *et al.* (2000). "Purification and biochemical characterization of a basic superantigen (SPEX/SMEZ3) from Streptococcus pyogenes." FEMS Microbiology Letters **188**(2): 153-63.

78. Gerlach, D., K. H. Schmidt, *et al.* (2001). "Basic streptococcal superantigens (SPEX/SMEZ or SPEC) are responsible for the mitogenic activity of the so-called mitogenic factor (MF)." FEMS Immunology and Medical Microbiology **30**(3): 209-16.

79. Gorga, J. C., V. Horejsi, *et al.* (1987). "Purification and characterization of class II histocompatibility antigens from a homozygous human B cell line." Journal of Biological Chemistry **262**(33): 16087-94.

80. Gu, J., B. Yu, *et al.* (1997). "[HLA-DQA1 genes involved in genetic susceptibility to rheumatic fever and rheumatic heart disease in southern Hans]." Zhonghua Nei Ke Za Zhi **36**(5): 308-11.

81. Guilherme, L., W. Weidebach, *et al.* (1991). "Association of human leukocyte class II antigens with rheumatic fever or rheumatic heart disease in a Brazilian population." Circulation **83**(6): 1995-8.

82. Hackett, S. P. and D. L. Stevens (1993). "Superantigens associated with staphylococcal and streptococcal toxic shock syndrome are potent inducers of tumor necrosis factor-beta synthesis." Journal of Infectious Diseases **168**(1): 232-5.

83. Haffner, A. C., K. Zepter, *et al.* (1996). "Major histocompatibility complex class I molecule serves as a ligand for presentation of the superantigen staphylococcal enterotoxin B to T cells." Proceedings of The National Academy of Sciences of the United States of America **93**(7): 3037-42.

84. Hajjeh, R. A., A. Reingold, *et al.* (1999). "Toxic shock syndrome in the United States: surveillance update, 1979 1996." Emerging Infectious Diseases **5**(6): 807-10.

85. Happ, M. P., D. L. Woodland, *et al.* (1989). "A third T-cell receptor beta-chain variable region gene encodes reactivity to Mls-1a gene products." Proceedings of

The National Academy of Sciences of the United States of America **86**(16): 6293-6.

86. Hargreaves, R. E., R. D. Brehm, *et al.* (1995). "Definition of sites on HLA-DR1 involved in the T cell response to staphylococcal enterotoxins E and C2." European Journal of Immunology **25**(12): 3437-44.

87. Harris, T. O., D. Grossman, *et al.* (1993). "Lack of complete correlation between emetic and T-cell-stimulatory activities of staphylococcal enterotoxins." Infection and Immunity **61**(8): 3175-83.

88. Hennecke, J. and D. C. Wiley (2001). "T cell receptor-MHC interactions up close." Cell **104**(1): 1-4.

89. Herman, A., G. Croteau, *et al.* (1990). "HLA-DR Alleles Differ in Their Ability to Present Staphylococcal Enterotoxins to T Cells." Journal of Experimental Medicine **172**: 706-717.

90. Herman, A., J. W. Kappler, *et al.* (1991). "Superantigens: mechanism of T-cell stimulation and role in immune responses." Annual Review of Immunology **9**: 745-72.

91. Herman, A., N. Labrecque, *et al.* (1991). "Identification of the staphylococcal enterotoxin A superantigen binding site in the beta 1 domain of the human histocompatibility antigen HLA-DR." Proceedings of The National Academy of Sciences of the United States of America **88**(22): 9954-8.

92. Hill, A. V. (1998). "The immunogenetics of human infectious diseases." Annual Review of Immunology **16**: 593-617.

93. Hodtsev, A. S., Y. Choi, *et al.* (1998). "Mycoplasma superantigen is a CDR3-dependent ligand for the T cell antigen receptor." Journal of Experimental Medicine **187**(3): 319-27.

94. Hogan, R. J., J. VanBeek, *et al.* (2001). "Identification of MHC Class II-Associated Peptides That Promote the Presentation of Toxic Shock Syndrome Toxin-1 to T Cells." Journal of Immunology **166**(11): 6514-6522.

95. Holm, S. E., A. Norrby, *et al.* (1992). "Aspects of pathogenesis of serious group A streptococcal infections in Sweden, 1988-1989." Journal of Infectious Diseases **166**(1): 31-7.

96. Hong-Geller, E. and G. Gupta (2003). "Therapeutic approaches to superantigen-based diseases: a review." Journal of Molecular Recognition **16**(2): 91-101.

97. Huber, B. T., P. N. Hsu, *et al.* (1996). "Virus-encoded superantigens." Microbiology Reviews **60**(3): 473-82.

98. Hudson, K. R., R. E. Tiedemann, *et al.* (1995). "Staphylococcal enterotoxin A has two cooperative binding sites on major histocompatibility complex class II." Journal of Infectious Diseases **182**(3): 711-20.

99. Hume, C. R. and J. S. Lee (1990). "Genetics of HLA class II regulation." Immunology Research **9**(2): 93-102.

100. Imanishi, K., H. Igarashi, *et al.* (1990). "Activation of murine T cells by streptococcal pyrogenic exotoxin type A. Requirement for MHC class II molecules on accessory cells and identification of V beta elements in T cell receptor of toxin-reactive T cells." Journal of Immunology **145**(10): 3170-6.

101. Imanishi, K., H. Igarashi, *et al.* (1992). "Relative abilities of distinct isotypes of human major histocompatibility complex class II molecules to bind streptococcal pyrogenic exotoxin types A and B." Infection and Immunity **60**(12): 5025-9.

102. Ito, Y., G. Seprenyi, *et al.* (1999). "Analysis of functional regions of YPM, a superantigen derived from gram-negative bacteria." European Journal of Biochemistry **263**(2): 326-37.

103. Janeway, C. A., Jr. (1991). "Selective elements for the V beta region of the T cell receptor: Mls and the bacterial toxic mitogens." Advances in Immunoloy **50**: 1-53.

104. Janeway, C. A., Jr., J. Yagi, *et al.* (1989). "T-cell responses to Mls and to bacterial proteins that mimic its behavior." Immunological Reviews **107**: 61-88.

105. Jardetzky, T. S., J. H. Brown, *et al.* (1994). "Three-dimensional structure of a human class II histocompatibility molecule complexed with superantigen." Nature **368**(6473): 711-8.

106. Johnson, D. R., J. T. Wotton, *et al.* (2002). "A comparison of group A streptococci from invasive and uncomplicated infections: are virulent clones responsible for serious streptococcal infections?" Journal of Infectious Diseases **185**(11): 1586-95.

107. Kappler, J., B. Kotzin, *et al.* (1989). "V beta-specific stimulation of human T cells by staphylococcal toxins." Science **244**(4906): 811-3.

108. Kappler, J. W., N. Roehm, *et al.* (1987). "T cell tolerance by clonal elimination in the thymus." Cell **49**(2): 273-80.

109. Kappler, J. W., U. Staerz, *et al.* (1988). "Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex." Nature **332**(6159): 35-40.

110. Karp, D. R. and E. O. Long (1992). "Identification of HLA-DR1 beta chain residues critical for binding staphylococcal enterotoxins A and E." Journal of Experimental Medicine **175**(2): 415-24.

111. Kato, S., M. Kimura, *et al.* (1978). "HLA antigens in Kawasaki disease." Pediatrics. **61**(2): 252-5.

112. Keren, G., Y. L. Danon, *et al.* (1982). "HLA Bw51 is increased in mucocutaneous lymph node syndrome in Israeli patients." Tissue Antigens. **20**(2): 144-6.

113. Kerlero de Rosbo, N., R. Milo, *et al.* (1993). "Reactivity to myelin antigens in multiple sclerosis. Peripheral blood lymphocytes respond predominantly to myelin oligodendrocyte glycoprotein." Journal of Clinical Investigation **92**(6): 2602-8.

114. Kim, J., R. G. Urban, *et al.* (1994). "Toxic shock syndrome toxin-1 complexed with a class II major histocompatibility molecule HLA-DR1." Science **266**(5192): 1870-4.

115. Kline, J. B. and C. M. Collins (1996). "Analysis of the superantigenic activity of mutant and allelic forms of streptococcal pyrogenic exotoxin A." Infection and Immunity **64**(3): 861-9.

116. Kline, J. B. and C. M. Collins (1997). "Analysis of the interaction between the bacterial superantigen streptococcal pyrogenic exotoxin A (SpeA) and the human T-cell receptor." Molecular Microbiology **24**(1): 191-202.

117. Kotb, M., A. Norrby-Teglund, *et al.* (2002). "An immunogenetic and molecular basis for differences in outcomes of invasive group A streptococcal infections." Nature Medicine **8**(12): 1398-1404.

118. Kotzin, B. L., D. Y. Leung, *et al.* (1993). "Superantigens and their potential role in human disease." Advances in Immunology **54**: 99-166.

119. Kozono, H., D. Parker, *et al.* (1995). "Multiple binding sites for bacterial superantigens on soluble class II MHC molecules." Immunity **3**(2): 187-96.

120. Krensky, A. M., W. Berenberg, *et al.* (1981). "HLA antigens in mucocutaneous lymph node syndrome in New England." Pediatrics. **67**(5): 741-3.

121. Kwok, W. W., S. Kovats, *et al.* (1993). "HLA-DQ allelic polymorphisms constrain patterns of class II heterodimer formation." Journal of Immunology **150**(6): 2263-72.

122. Lafon, M., E. Jouvin-Marche, *et al.* (2002). "Human viral superantigens: to be or not to be transactivated?" Trends in Immunology **23**(5): 238-9; author reply 239.

123. Lafon, M., M. Lafage, *et al.* (1992). "Evidence for a viral superantigen in humans." Nature **358**(6386): 507-10.

124. Lafon, M., D. Scott-Algara, *et al.* (1994). "Neonatal deletion and selective expansion of mouse T cells by exposure to rabies virus nucleocapsid superantigen." Journal of Experimental Medicine **180**(4): 1207-15.

125. Lamphear, J. G., K. R. Stevens, *et al.* (1998). "Intercellular adhesion molecule-1 and leukocyte function-associated antigen-3 provide costimulation for

superantigen-induced T lymphocyte proliferation in the absence of a specific presenting molecule." Journal of Immunology **160**(2): 615-23.

126. Lampson, L. A. and R. Levy (1980). "Two populations of Ia-like molecules on a human B cell line." Journal of Immunology **125**(1): 293-9.

127. Lando, P., C. Olsson, *et al.* (1996). "Regulation of Superantigen induced T-cell activation in the absence and the presence of MHC class II." Journal of Immunolgy **157**: 2857-2863.

128. Lanzavecchia, A. (1996). "Mechanisms of antigen uptake for presentation." Current Opinion in Immunology **8**(3): 348-54.

129. Lavoie, P. M., H. McGrath, *et al.* (2001). "Quantitative relationship between MHC class II-superantigen complexes and the balance of T cell activation versus death." Journal of Immunology **166**(12): 7229-37.

130. Lavoie, P. M., J. Thibodeau, *et al.* (1997). "Selective binding of bacterial toxins to major histocompatibility complex class II-expressing cells is controlled by invariant chain and HLA-DM." Proceedings of The National Academy of Sciences of the United States of America **94**(13): 6892-7.

131. Leder, L., A. Llera, *et al.* (1998). "A mutational analysis of the binding of staphylococcal enterotoxins B and C3 to the T cell receptor beta chain and major histocompatibility complex class II." Journal of Experimental Medicine **187**(6): 823-33.

132. Leung, D. Y., M. Gately, *et al.* (1995). "Bacterial superantigens induce T cell expression of the skin-selective homing receptor, the cutaneous lymphocyte-associated antigen, via stimulation of interleukin 12 production." Journal of Experimental Medicine **181**(2): 747-53.

133. Leung, D. Y., C. Meissner, *et al.* (1995). "The potential role of bacterial superantigens in the pathogenesis of Kawasaki syndrome." Journal of Clinical Immunology **15**(6 Suppl): 11S-17S.

134. Leung, D. Y., J. B. Travers, *et al.* (1995). "Evidence for a streptococcal superantigen-driven process in acute guttate psoriasis." Journal of Clinical Investigation **96**(5): 2106-12.

135. Li, H., A. Llera, *et al.* (1999). "The structural basis of T cell activation by superantigens." Annual Review of Immunology **17**: 435-66.

136. Li, H., A. Llera, *et al.* (1998). "Structure-function studies of T-cell receptor-superantigen interactions." Immunological Reviews **163**: 177-86.

137. Li, H., A. Llera, *et al.* (1998). "Three-dimensional structure of the complex between a T cell receptor beta chain and the superantigen staphylococcal enterotoxin B." Immunity **9**(6): 807-16.

138. Li, Y., H. Li, *et al.* (2001). "Crystal structure of a superantigen bound to the high-affinity, zinc-dependent site on MHC class II." Immunity **14**(1): 93-104.

139. Llewelyn, M. and J. Cohen (2002). "Superantigens: microbial agents that corrupt immunity." Lancet Infectious Diseases **2**(3): 156-62.

140. Lotteau, V., L. Teyton, *et al.* (1987). "A novel HLA class II molecule (DR alpha-DQ beta) created by mismatched isotype pairing." Nature **329**(6137): 339-41.

141. Luther, S. A. and H. Acha-Orbea (1997). "Mouse mammary tumor virus: immunological interplays between virus and host." Advances in Immunology **65**: 139-243.

142. MacDonald, H. R., S. Baschieri, *et al.* (1991). "Clonal expansion precedes anergy and death of V beta 8+ peripheral T cells responding to staphylococcal enterotoxin B in vivo." European Journal of Immunology **21**(8): 1963-6.

143. MacDonald, H. R., R. Schneider, *et al.* (1988). "T-cell receptor V beta use predicts reactivity and tolerance to Mlsa-encoded antigens." Nature **332**(6159): 40-5.

144. Malchiodi, E. L., E. Eisenstein, *et al.* (1995). "Superantigen binding to a T cell receptor beta chain of known three-dimensional structure." Journal of Experimental Medicine **182**(6): 1833-45.

145. Marrack, P., M. Blackman, *et al.* (1990). "The toxicity of staphylococcal enterotoxin B in mice is mediated by T cells." Journal of Experimental Medicine **171**(2): 455-64.

146. Marrack, P. and J. Kappler (1990). "The staphylococcal enterotoxins and their relatives." Science **248**(4956): 705-11.

147. Marsh, S. G., P. Parham, *et al.* (2000). Part 10 HLA DQ. The HLA Facts Book. London, Academic Press**:** 304-329.

148. Martinez-Arends, A., E. Astoul, *et al.* (1995). "Activation of human tonsil lymphocytes by rabies virus nucleocapsid superantigen." Clinical Immunology and Immunopathology 77(2): 177-84.

149. Mathis, D. J., C. Benoist, *et al.* (1983). "Several mechanisms can account for defective E alpha gene expression in different mouse haplotypes." Proceedings of The National Academy of Sciences of the United States of America **80**(1): 273-7.

150. Matsuda, I., S. Hattori, *et al.* (1977). "HLA antigens in mucocutaneous lymph node syndrome." American Journal of Diseases of Children. **131**(12): 1417-8.

151. McCormick, J. K., J. M. Yarwood, *et al.* (2001). "Toxic shock syndrome and bacterial superantigens: an update." Annual Review of Microbiology **55**: 77-104.

152. Meinl, E. and R. Hohlfeld (2002). "Immunopathogenesis of multiple sclerosis: MBP and beyond." Clinical and Experimental Immunology **128**(3): 395-7.

153. Michie, C., A. Scott, *et al.* (1994). "Streptococcal toxic shock like syndrome; evidence of superantigen activity and its effects on T lymphocyte subsets in vitro." Clinical and Experimental Immunology **98**: 140-144.

154. Michie, C. A. and T. Davis (1996). "Atopic dermatitis and staphylococcal superantigens." Lancet **347**(8997): 324.

155. Micusan, V. V. and J. Thibodeau (1993). "Superantigens of microbial origin." Seminares in Immunology **5**(1): 3-11.

156. Miethke, T., K. Duschek, *et al.* (1993). "Pathogenesis of the toxic shock syndrome: T cell mediated lethal shock caused by the superantigen TSST-1." European Journal of Immunology **23**(7): 1494-500.

157. Miethke, T., C. Wahl, *et al.* (1992). "T cell-mediated lethal shock triggered in mice by the superantigen staphylococcal enterotoxin B: critical role of tumor necrosis factor." Journal of Experimental Medicine **175**(1): 91-8.

158. Miethke, T., C. Wahl, *et al.* (1993). "Superantigen mediated shock: a cytokine release syndrome." Immunobiology **189**(3-4): 270-84.

159. Mitchell, D. T., D. G. Levitt, *et al.* (2000). "Structural evidence for the evolution of pyrogenic toxin superantigens." Journal of Molecular Evolution **51**(6): 520-31.

160. Miyoshi-Akiyama, T., J. Zhao, *et al.* (2003). "Streptococcus dysgalactiae-derived mitogen (SDM), a novel bacterial superantigen: characterization of its biological activity and predicted tertiary structure." Molecular Microbiology **47**(6): 1589-99.

161. Mollick, J. A., M. Chintagumpala, *et al.* (1991). "Staphylococcal exotoxin activation of T cells. Role of exotoxin-MHC class II binding affinity and class II isotype." Journal of Immunology **146**(2): 463-8.

162. Muller-Alouf, H., J. E. Alouf, *et al.* (1992). "Cytokine production by murine cells activated by erythrogenic toxin type A superantigen of Streptococcus pyogenes." Immunobiology **186**(5): 435-48.

163. Murphy, K. M., A. B. Heimberger, *et al.* (1990). "Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo." Science **250**(4988): 1720-3.

164. Murzin, A. G. (1993). "OB(oligonucleotide/oligosaccharide binding)-fold: common structural and functional solution for non-homologous sequences." EMBO Journal **12**(3): 861-7.

165. Murzin, A. G., S. E. Brenner, *et al.* (1995). "SCOP: a structural classification of proteins database for the investigation of sequences and structures." Journal of Molecular Biology **247**(4): 536-40.

166. Musser, J. M., A. R. Hauser, *et al.* (1991). "Streptococcus pyogenes causing toxic-shock-like syndrome and other invasive diseases: clonal diversity and pyrogenic exotoxin expression." Proceedings of The National Academy of Sciences of the United States of America **88**(7): 2668-72.

167. Musser, J. M., V. Kapur, *et al.* (1993). "Geographic and temporal distribution and molecular characterization of two highly pathogenic clones of Streptococcus pyogenes expressing allelic variants of pyrogenic exotoxin A (Scarlet fever toxin)." Journal of Infectious Diseases **167**(2): 337-46.

168. Nelson, K., P. M. Schlievert, *et al.* (1991). "Characterization and clonal distribution of four alleles of the *speA* gene encoding pyrogenic exotoxin A (scarlet fever toxin) in *Streptococcus pyogenes*." Journal of Experimental Medicine **174**: 1271-1274.

169. Newton, D. W., M. Dohlsten, *et al.* (1996). "Mutations in the MHC class II binding domains of staphylococcal enterotoxin A differentially affect T cell receptor Vbeta specificity." Journal of Immunology **157**(9): 3988-94.

170. Norrby-Teglund, A., G. T. Nepom, *et al.* (2002). "Differential presentation of group A streptococcal superantigens by HLA class II DQ and DR alleles." European Journal of Immunology **32**: 2570-77.

171. Oliveira, D. B. (1997). "Poststreptococcal glomerulonephritis: getting to know an old enemy." Clinical and Experimental Immunology **107**(1): 8-10.

172. Olmez, U., M. Turgay, *et al.* (1993). "Association of HLA class I and class II antigens with rheumatic fever in a Turkish population." Scandinavian Journal of Immunology **22**(2): 49-52.

173. Ota, K., M. Matsui, *et al.* (1990). "T-cell recognition of an immunodominant myelin basic protein epitope in multiple sclerosis." Nature **346**(6280): 183-7.

174. Papageorgiou, A. C. and K. R. Acharya (1997). "Superantigens as immunomodulators: recent structural insights." Structure **5**(8): 991-6.

175. Papageorgiou, A. C., C. M. Collins, *et al.* (1999). "Structural basis for the recognition of superantigen streptococcal pyrogenic exotoxin A (SpeA1) by MHC class II molecules and T-cell receptors." EMBO Journal **18**(1): 9-21.

176. Papageorgiou, A. C., H. S. Tranter, *et al.* (1998). "Crystal structure of microbial superantigen staphylococcal enterotoxin B at 1.5 A resolution: implications for superantigen recognition by MHC class II molecules and T-cell receptors." Journal of Molecular Biology **277**(1): 61-79.

177. Perron, H., E. Jouvin-Marche, *et al.* (2001). "Multiple sclerosis retrovirus particles and recombinant envelope trigger an abnormal immune response in vitro, by inducing polyclonal Vbeta16 T-lymphocyte activation." Virology **287**(2): 321-32.

178. Petersson, K., M. Hakansson, *et al.* (2001). "Crystal structure of a superantigen bound to MHC class II displays zinc and peptide dependence." EMBO Journal **20**(13): 3306-12.

179. Petersson, K., H. Pettersson, *et al.* (2003). "Staphylococcal enterotoxin H induces valpha-specific expansion of T cells." Journal of Immunology **170**(8): 4148-54.

180. Petersson, K., M. Thunnissen, *et al.* (2002). "Crystal structure of a SEA variant in complex with MHC class II reveals the ability of SEA to crosslink MHC molecules." Structure (Cambridge) **10**(12): 1619-26.

181. Plano, L. R., D. M. Gutman, *et al.* (2000). "Recombinant Staphylococcus aureus exfoliative toxins are not bacterial superantigens." Infection and Immunity **68**(5): 3048-52.

182. Proft, T. and J. Fraser (2003). "Bacterial Superantigens." Clinical and Experimental Immunology **133**(3): 209-306.

183. Proft, T., S. L. Moffatt, *et al.* (2000). "The streptococcal superantigen SMEZ exhibits wide allelic variation, mosaic structure, and significant antigenic variation." Journal of Experimental Medicine **191**(10): 1765-76.

184. Proft, T., S. Sriskandan, *et al.* (2003). "Superantigens and Streptococcal Toxic Shock Syndrome." Emerging Infectious Diseases **9**(10).

185. Proft, T., P. D. Webb, *et al.* (2003). "Two novel superantigens found in both group A and group C Streptococcus." Infection and Immunity **71**(3): 1361-9.

186. Pullen, A. M., J. Bill, *et al.* (1991). "Analysis of the interaction site for the self superantigen Mls-1a on T cell receptor V beta." Journal of Experimental Medicine **173**(5): 1183-92.

187. Pullen, A. M., P. Marrack, *et al.* (1988). "The T-cell repertoire is heavily influenced by tolerance to polymorphic self-antigens." Nature **335**(6193): 796-801.

188. Pullen, A. M., T. Wade, *et al.* (1990). "Identification of the region of T cell receptor beta chain that interacts with the self-superantigen MIs-1a." Cell **61**(7): 1365-74.

189. Rajagopalan, G., M. K. Smart, *et al.* (2002). "Staphylococcal enterotoxin B-induced activation and concomitant resistance to cell death in CD28-deficient HLA-DQ8 transgenic mice." International Immunology **14**(7): 801-12.

190. Reinherz, E. L., K. Tan, *et al.* (1999). "The crystal structure of a T cell receptor in complex with peptide and MHC class II." Science **286**(5446): 1913-21.

191. Reith, W., E. Barras, *et al.* (1989). "Cloning of the major histocompatibility complex class II promoter binding protein affected in a hereditary defect in class II gene regulation." Proceedings of The National Academy of Sciences of the United States of America **86**(11): 4200-4.

192. Robinson, J., M. Waller, *et al.* (2003). "IMGT/HLA and IMGT/MHC: sequence databases for the study of the major histocompatibility complex." Nucleic Acids Research **31**: 311-314.

193. Robinson, K. A., G. Rothrock, *et al.* (2003). "Risk for severe group A streptococcal disease among patients' household contacts." Emerging Infectious Diseases **9**(4): 443-7.

194. Roussel, A., B. F. Anderson, *et al.* (1997). "Crystal structure of the streptococcal superantigen SPE-C: dimerization and zinc binding suggest a novel mode of interaction with MHC class II molecules." Nature Structural Biology **4**(8): 635-43.

195. Rowen, L., B. F. Koop, *et al.* (1996). "The complete 685-kilobase DNA sequence of the human beta T cell receptor locus." Science **272**(5269): 1755-62.

196. Rudge, P. (1991). "Does a retrovirally encoded superantigen cause multiple sclerosis?" Journal of Neurology, Neurosurgery and Psychiatry **54**(10): 853-5.

197. Saha, B., D. M. Harlan, *et al.* (1996). "Protection against lethal toxic shock by targeted disruption of the CD28 gene." Journal of Experimental Medicine **183**(6): 2675-80.

198. Schad, E. M., I. Zaitseva, *et al.* (1995). "Crystal structure of the superantigen staphylococcal enterotoxin type A." EMBO Journal **14**(14): 3292-301.

199. Schlievert, P. M. (1982). "Enhancement of host susceptibility to lethal endotoxin shock by staphylococcal pyrogenic exotoxin type C." Infection and Immunity **36**(1): 123-8.

200. Schlievert, P. M. (1993). "Role of superantigens in human disease." Journal of Infectious Diseases **167**(5): 997-1002.

201. Schlievert, P. M. and E. D. Gray (1989). "Group A streptococcal pyrogenic exotoxin (scarlet fever toxin) type A and blastogen A are the same protein." Infection and Immunity **57**(6): 1865-7.

202. Schlievert, P. M., L. M. Jablonski, *et al.* (2000). "Pyrogenic toxin superantigen site specificity in toxic shock syndrome and food poisoning in animals." Infection and Immunity **68**(6): 3630-4.

203. Scholl, P. R., A. Diez, *et al.* (1990). "Effect of isotypes and allelic polymorphism on the binding of staphylococcal exotoxins to MHC class II molecules." Journal of Immunology **144**(1): 226-30.

204. Seth, A., L. J. Stern, *et al.* (1994). "Binary and ternary complexes between T-cell receptor, class II MHC and superantigen in vitro." Nature **369**(6478): 324-7.

205. Sherman, L. A. and S. Chattopadhyay (1993). "The molecular basis of allorecognition." Annual Review of Immunology **11**: 385-402.

206. Shimonkevitz, R., J. Kappler, *et al.* (1983). "Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing." Journal of Experimental Medicine **158**(2): 303-16.

207. Shupp, J. W., M. Jett, *et al.* (2002). "Identification of a transcytosis epitope on staphylococcal enterotoxins." Infection and Immunity **70**(4): 2178-86.

208. Sigrist, C. J., L. Cerutti, *et al.* (2002). "PROSITE: a documented database using patterns and profiles as motif descriptors." Briefings in Bioinformatics **3:**: 265-274.

209. Smoot, J. C., K. D. Barbian, *et al.* (2002). "Genome sequence and comparative microarray analysis of serotype M18 group A Streptococcus strains associated with acute rheumatic fever outbreaks." Proceedings of The National Academy of Sciences of the United States of America **99**(7): 4668-73.

210. Smoot, L. M., J. K. McCormick, *et al.* (2002). "Characterization of two novel pyrogenic toxin superantigens made by an acute rheumatic fever clone of Streptococcus pyogenes associated with multiple disease outbreaks." Infection and Immunity **70**(12): 7095-104.

211. Spits, H., J. Borst, *et al.* (1984). "HLA-DC antigens can serve as recognition elements for human cytotoxic T lymphocytes." European Journal of Immunology **14**(4): 299-304.

212. Sriskandan, S., D. Moyes, *et al.* (1996). "Streptococcal pyrogenic exotoxin A release, distribution, and role in a murine model of fasciitis and multiorgan failure due to Streptococcus pyogenes." Journal of Infectious Diseases **173**(6): 1399-407.

213. Sriskandan, S., D. Moyes, *et al.* (1996). "Detection of circulating bacterial superantigen and lymphotoxin-alpha in patients with streptococcal toxic-shock syndrome." Lancet **348**(9037): 1315-6.

214. Sriskandan, S., M. Unnikrishnan, *et al.* (1999). "Molecular analysis of the role of streptococcal pyrogenic Exotoxin A (SPEA) in invasive soft-tissue infection resulting from Streptococcus pyogenes." Molecular Microbiology **33**(4): 778-90.

215. Sriskandan, S., M. Unnikrishnan, *et al.* (2000). "Mitogenic factor (MF) is the major DNase of serotype M89 streptococcus pyogenes." Microbiology **146**(Pt 11): 2785-92.

216. Sriskandan, S., M. Unnikrishnan, *et al.* (2001). "Enhanced susceptibility to superantigen-associated streptococcal sepsis in human leukocyte antigen-DQ transgenic mice." Journal of Infectious Diseases **184**(2): 166-73.

217. Stauffer, Y., S. Marguerat, *et al.* (2001). "Interferon-alpha-induced endogenous superantigen. a model linking environment and autoimmunity." Immunity **15**(4): 591-601.

218. Stevens, D. L. (1994). "Invasive group A streptococcal infections: the past, present and future." Pediatric Infectious Diseases Journal **13**(6): 561-6.

219. Stevens, D. L. (1996). "The toxic shock syndromes." Infectious Disease Clinics of North America **10**(4): 727-46.

220. Stevens, D. L., A. E. Bryant, *et al.* (1996). "Group A streptococcal bacteremia: the role of tumor necrosis factor in shock and organ failure." Journal of Infectious Diseases **173**(3): 619-26.

221. Stollerman, G. H. (2001). "Rheumatic fever in the 21st century." Clinical Infectious Diseases **33**(6): 806-14.

222. Stolz, S. J., J. P. Davis, *et al.* (1985). "Development of serum antibody to toxic shock toxin among individuals with toxic shock syndrome in Wisconsin." Journal of Infectious Diseases **151**(5): 883-9.

223. Sun, J. B., T. Olsson, *et al.* (1991). "Autoreactive T and B cells responding to myelin proteolipid protein in multiple sclerosis and controls." European Journal of Immunology **21**(6): 1461-8.

224. Sundberg, E. and T. S. Jardetzky (1999). "Structural basis for HLA-DQ binding by the streptococcal superantigen SSA." Nature Structural Biology **6**(2): 123-9.

225. Sundberg, E. J., H. Li, *et al.* (2002). "Structures of Two Streptococcal Superantigens Bound to TCR beta Chains Reveal Diversity in the Architecture of T Cell Signaling Complexes." Structure (Cambridge) **10**(5): 687-99.

226. Sundstrom, M., L. Abrahmsen, *et al.* (1996). "The crystal structure of staphylococcal enterotoxin type D reveals Zn2+-mediated homodimerization." EMBO Journal **15**(24): 6832-40.

227. Surman, S., A. M. Deckhut, *et al.* (1994). "MHC-specific recognition of a bacterial superantigen by weakly reactive T cells." Journal of Immunology **152**(10): 4893-902.

228. Sutkowski, N., B. Conrad, *et al.* (2001). "Epstein-Barr Virus Transactivates the Human Endogenous Retrovirus HERV-K18 that Encodes a Superantigen." Immunity **15**(4): 579-89.

229. Sutkowski, N., T. Palkama, *et al.* (1996). "An Epstein-Barr virus-associated superantigen." Journal of Experimental Medicine **184**(3): 971-80.

230. Suzuki, H., Y. Muragaki, *et al.* (2002). "Detection of auto-antibodies against a 70 kDa protein derived from vascular smooth muscle cells in patients with Kawasaki disease." European Journal of Pediatrics **161**(6): 324-9.

231. Talkington, D. F., B. Schwartz, *et al.* (1993). "Association of phenotypic and genotypic characteristics of invasive Streptococcus pyogenes isolates with clinical components of streptococcal toxic shock syndrome." Infection and Immunity **61**(8): 3369-74.

232. Telfer, N. R., R. J. Chalmers, *et al.* (1992). "The role of streptococcal infection in the initiation of guttate psoriasis." Archives of Dermatology **128**(1): 39-42.

233. Tiedemann, R. E., R. J. Urban, *et al.* (1995). "Isolation of HLA-DR1.(staphylococcal enterotoxin A)2 trimers in solution." Proceedings of The National Academy of Sciences of the United States of America **92**(26): 12156-9.

234. Todd, J., M. Fishaut, *et al.* (1978). "Toxic-shock syndrome associated with phage-group-I Staphylococci." Lancet **2**(8100): 1116-8.

235. Tomai, M. A., P. M. Schlievert, *et al.* (1992). "Distinct T-cell receptor V beta gene usage by human T lymphocytes stimulated with the streptococcal pyrogenic exotoxins and pep M5 protein." Infection and Immunity **60**(2): 701-5.

236. Uchiyama, T., K. Imanishi, *et al.* (1989). "Activation of human T cells by toxic shock syndrome toxin-1: the toxin- binding structures expressed on human lymphoid cells acting as accessory cells are HLA class II molecules." European Journal of Immunology **19**(10): 1803-9.

237. Unnikrishnan, M., J. Cohen, *et al.* (1999). "Growth-phase-dependent expression of virulence factors in an M1T1 clinical isolate of Streptococcus pyogenes." Infection and Immunity **67**(10): 5495-9.

238. van Rood, J. J. (1993). "HLA and I." Annual Review of Immunology **11**: 1-28.

239. Visentainer, J. E., F. C. Pereira, *et al.* (2000). "Association of HLA-DR7 with rheumatic fever in the Brazilian population." Journal of Rheumatology **27**(6): 1518-20.

240. Visvanathan, K., A. Charles, *et al.* (2001). "Inhibition of Bacterial Superantigens by Peptides and Antibodies." Infection and Immunity **69**(2): 875-884.

241. von Bonin, A., S. Ehrlich, *et al.* (1995). "Major histocompatibility complex class II-associated peptides determine the binding of the superantigen toxic shock syndrome toxin-1." European Journal of Immunology **25**(10): 2894-8.

242. Wahl, C., T. Miethke, *et al.* (1993). "Clonal deletion as direct consequence of an in vivo T cell response to bacterial superantigen." European Journal of Immunology **23**(5): 1197-200.

243. Wantanabe-Ohnishi, R., D. Low, *et al.* (1995). "Selective Depletion of Vβ-Bearing T Cells in Patients with Severe Invasive Group A Streptococcal Infections and Streptococcal Toxic Shock Syndrome." Journal of Infectious Diseases **171**: 74-84.

244. Wei, S., P. Charmley, *et al.* (1994). "The extent of the human germline T-cell receptor V beta gene segment repertoire." Immunogenetics **40**(1): 27-36.

245. Wen, R., M. A. Blackman, *et al.* (1995). "Variable influence of MHC polymorphism on the recognition of bacterial superantigens by T cells." Journal of Immunology **155**(4): 1884-92.

246. White, J., A. Herman, *et al.* (1989). "The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice." Cell **56**(1): 27-35.

247. Wilson, R., K., E. Lai, *et al.* (1988). "Structure, Organisation and Polymorphism of murine and human T-cell receptor α and β chain gene families." Immunological Reviews **101**: 149-172.

248. Woodland, D. L. (2002). "Immunity and retroviral superantigens in humans." Trends in Immunology **23**(2): 57-8.

249. Wright, A. D. and S. K. Chapes (1999). "Cross-linking staphylococcal enterotoxin A bound to major histocompatibility complex class I is required for TNF-alpha secretion." Cellular Immunology **197**(2): 129-35.

250. Wucherpfennig, K. W. (2001). "Mechanisms for the induction of autoimmunity by infectious agents." Journal of Clinical Investigation **108**(8): 1097-104.

251. Yeung, R., J. Penninger, *et al.* (1996). "Human CD4 and human MHC class II (DQ6) transgenic mice: supersensitivity to superantigen-induced septic shock." European Journal of Immunology **26**: 1074-1082.

252. Zhang, J., C. Vandevyver, *et al.* (1995). "Activation and clonal expansion of human myelin basic protein-reactive T cells by bacterial superantigens." Journal of Autoimmunity **8**(4): 615-32.

253. Zhou, P., M. K. Smart, *et al.* (1992). "HLA-DQ beta chain can present mouse endogenous provirus MTV-9 product and clonally delete Tcr V beta 5+ and V beta 11+ T cells in transgenic mice." Immunogenetics **35**(4): 219-23.

Appendix 1. TCRVbeta aminoacid sequences. Numbering as for mVB8.2 (Folch and Lefranc 2000; Arden, Clark *et al.* 1995)

TCRVbeta	*TRBV gene*	FR1	CDR1	FR2	CDR2	FR3	CDR3
		1	27	32	49	55	
mV8.2	*mTRBV13-2*	EAAVTQSPRNKVAVTGGKVTLSCNQT	NNHNN	MYWYRQDTGHGLRLIHY	SYGAGS	TEKGDIP.DGYKASRP.S	
22	*TRBV2(1)*	EPEVTQTPSHQVTQMGQEVILRCVPI	SNHLY....	FYWYRQILGQKVEFLVS	FYNNEI..	SEKSEIFDDQFSVERP.DGSNFTLKIRSTKLEDSAMYFC	ASSE.....
9.1	*TRBV3-1*	DTAVSQTPKYLVTQMGNDKSIKCEQN	LGHDT....	MYWYKQDSKKFLKIMFS	YNNKEL..	IINETVP.NRFSPKSP.DKAHLNLHINSLELGDSAVYFC	ASSQ.....
7.1	*TRBV4-1*	DTEVTQTPKHLVMGMTNKKSLKCEQH	MGHRA....	MYWYKQKAKKPPELMFV	YSYEKL..	SINESVP.SRFSPECP.NSSLLNLHLHALQPEDSALYLC	ASSQ.....
7.3	*TRBV4-2*	ETGVTQTPRHLVMGMTNKKSLKCEQH	LGHNA....	MYWYKQSAKKPLELMFV	YNFKEQ..	TENNSVP.SRFSPECP.NSSHLFLHLHTLQPEDSALYLC	ASSQ.....
7.2	*TRBV4-3*	ETGVTQTPRHLVMGMTNKKSLKCEQH	LGHNA....	MYWYKQSAKKPLELMFV	YSLEER..	VENNSVP.SRFSPECP.NSSHLFLHLHTLQPEDSALYLC	ASSQ.....
5.1	*TRBV5-1(1)*	KAGVTQTPRYLIKTRGQQVTLSCSPI	SGHRS....	VSWYQQTPGQGLQFLFE	YFSETQ..	RNKGNFP.GRFSGRQF.SNSRSEMNVSTLELGDSALYLC	ASSL.....
5.5	*TRBV5-3*	EAGVTQSPTHLIKTRGQQVTLRCSPI	SGHSS....	VSWYQQAPGQGPQFIFE	YANELR..	RSEGNFP.NRFSGRQF.HDCCSEMNVSALELGDSALYLC	ARSL.....
5.6	*TRBV5-4(1)*	ETGVTQSPTHLIKTRGQQVTLRCSSQ	SGHNT....	VSWYQQALGQGPQFIFQ	YYREEE..	NGRGNFP.PRFSGLQF.PNYSSELNVNALELDDSALYLC	ASSL.....
5.3	*TRBV5-5(1)*	DAGVTQSPTHLIKTRGQQVTLRCSPI	SGHKS....	VSWYQQVLGQGPQFIFQ	YYEKEE..	RGRGNFP.DRFSARQF.PNYSSELNVNALLLGDSALYLC	ASSL.....
5.2	*TRBV5-6(1)*	DAGVTQSPTHLIKTRGQQVTLRCSPK	SGHDT....	VSWYQQALGQGPQFIFQ	YYEEEE..	RQRGNFP.DRFSGHQF.PNYSSELNVNALLLGDSALYLC	ASSL.....
	TRBV5-7(1)	DAGVTQSPTHLIKTRGQHVTLRCSPI	SGHTS....	VSSYQQALGQGPQFIFQ	YYEKEE..	RGRGNFP.DQFSGHQF.PNYSSELNVNALLLGDSALYLC	ASSL.....
5.4	*TRBV5-8(1)*	EAGVTQSPTHLIKTRGQQATLRCSPI	SGHTS....	VYWYQQALGLGLQFLLW	YDEGEE..	RNRGNFP.PRFSGRQF.PNYSSELNVNALELEDSALYLC	ASSL.....
13.3	*TRBV6-1*	NAGVTQTPKFQVLKTGQSMTLQCAQD	MNHNS....	MYWYRQDPGMGLRLIYY	SASEGT..	TDKGEVP.NGYNVSRL.NKREFSLRLESAAPSQTSVYFC	ASSE.....
13.2a	*TRBV6-2*	NAGVTQTPKFRVLKTGQSMTLLCAQD	MNHEY....	MYWYRQDPGMGLRLIHY	SVGEGT..	TAKGEVP.DGYNVSRL.KKQNFLLGLESAAPSQTSVYFC	ASSY.....
13.2b	*TRBV6-3*	NAGVTQTPKFRVLKTGQSMTLLCAQD	MNHEY....	MYWYRQDPGMGLRLIHY	SVGEGT..	TAKGEVP.DGYNVSRL.KKQNFLLGLESAAPSQTSVYFC	ASSY.....
13.5	*TRBV6-4*	IAGITQAPTSQILAAGRRMTLRCTQD	MRHNA....	MYWYRQDLGLGLRLIHY	SNTAGT..	TGKGEVP.DGYSVSRA.NTDDFPLTLASAVPSQTSVYFC	ASSD.....
13.1	*TRBV6-5(1)*	NAGVTQTPKFQVLKTGQSMTLQCAQD	MNHEY....	MSWYRQDPGMGLRLIHY	SVGAGI..	TDQGEVP.NGYNVSRS.TTEDFPLRLLSAAPSQTSVYFC	ASSY.....
13.6	*TRBV6-6(1)*	NAGVTQTPKFRILKIGQSMTLQCTQD	MNHNY....	MYWYRQDPGMGLKLIYY	SVGAGI..	TDKGEVP.NGYNVSRS.TTEDFPLRLELAAPSQTSVYFC	ASSY.....
13.8	*TRBV6-7(1)*	NAGVTQTPKFHVLKTGQSMTLLCAQD	MNHEY....	MYRYRQDPGKGLRLIYY	SVAAAL..	TDKGEVP.NGYNVSRS.NTEDFPLKLESAAPSQTSVYFC	ASSY.....
13.7	*TRBV6-8(1)*	NAGVTQTPKFHILKTGQSMTLQCAQD	MNHGY....	MSWYRQDPGMGLRLIYY	SAAAGT..	TDK.EVP.NGYNVSRL.NTEDFPLRLVSAAPSQTSVYLC	ASSY.....
13.4	*TRBV6-9*	NAGVTQTPKFHILKTGQSMTLQCAQD	MNHGY....	LSWYRQDPGMGLRRIHY	SVAAGI..	TDKGEVP.DGYNVSRS.NTEDFPLRLESAAPSQTSVYFC	ASSY.....
6.7	*TRBV7-1*	GAGVSQSLRHKVAKKGKDVALRYDPI	SGHNA....	LYWYRQSLGQGLEFPIY	FQGKDA..	ADKSGLPRDRFSAQRS.EGSISTLKFQRTQQGDLAVYLC	ASSS.....
6.5	*TRBV7-2*	GAGVSQSPSNKVTEKGKDVELRCDPI	SGHTA....	LYWYRQSLGQGLEFLIY	FQGNSA..	PDKSGLPSDRFSAERT.GGSVSTLTIQRTQQEDSAVYLC	ASSL.....
6.1	*TRBV7-3*	GAGVSQTPSNKVTEKGKYVELRCDPI	SGHTA....	LYWYRQSLGQGPEFLIY	FQGTGA..	ADDSGLPNDRFFAVRP.EGSVSTLKIQRTERGDSAVYLC	ASSL.....
6.8	*TRBV7-4(1)*	GAGVSQSPRYKVAKRGRDVALRCDSI	SGHVT....	LYWYRQTLGQGSEVLTY	SQSDAQ..	RDKSGRPSGRFSAERP.ERSVSTLKIQRTEQGDSAVYLC	ASSL.....
6.3	*TRBV7-6(1)*	GAGVSQSPRYKVTKRGQDVALRCDPI	SGHVS....	LYWYRQALGQGPEFLTY	FNYEAQ..	QDKSGLPNDRFSAERP.EGSISTLTIQRTEQRDSAMYRC	ASSL.....
6.6	*TRBV7-7(1)*	GAGVSQSPRYKVTKRGQDVTLRCDPI	SSHAT....	LYWYQQALGQGPEFLTY	FNYEAQ..	PDKSGLPSDRFSAERP.EGSISTLTIQRTEQRDSAMYRC	ASSL.....
6.2	*TRBV7-8*	GAGVSQSPRYKVAKRGQDVALRCDPI	SGHVS....	LFWYQQALGQGPEFLTY	FQNEAQ..	LDKSGLPSDRFFAERP.EGSVSTLKIQRTQQEDSAVYLC	ASSL.....
6.4	*TRBV7-9(1)*	DTGVSQNPRHKITKRGQNVTFRCDPI	SEHNR....	LYWYRQTLGQGPEFLTY	FQNEAQ..	LEKSRLLSDRFSAERP.KGSFSTLEIQRTEQGDSAMYLC	ASSL.....
1	*TRBV9(1)*	DSGVTQTPKHLITATGQRVTLRCSPR	SGDLS....	VYWYQQSLDQGLQFLIQ	YYNGEE..	RAKGNIL.ERFSAQQF.PDLHSELNLSSLELGDSALYFC	ASSV.....
12.2	*TRBV10-1*	DAEITQSPRHKITETGRQVTLACHQT	WNHNN....	MFWYRQDLGHGLRLIHY	SYGVQD..	TNKGEVS.DGYSVSRS.NTEDLPLTLESAASSQTSVYFC	ASSE.....
12.3	*TRBV10-2*	DAGITQSPRYKITETGRQVTLMCHQT	WSHSY....	MFWYRQDLGHGLRLIYY	SAAADI..	TDKGEVP.DGYVVSRS.KTENFPLTLESATRSQTSVYFC	ASSE.....
12.1	*TRBV10-3*	DAGITQSPRHKVTETGTPVTLRCHQT	ENHRY....	MYWYRQDPGHGLRLIHY	SYGVKD..	TDKGEVS.DGYSVSRS.KTEDFLLTLESATSSQTSVYFC	AISE.....
21.1	*TRBV11-1*	EAEVAQSPRYKITEKSQAVAFWCDPI	SGHAT....	LYWYRQILGQGPELLVQ	FQDESV..	VDDSQLPKDRFSAERL.KGVDSTLKIQPAELGDSAMYLC	ASSL.....
21.3	*TRBV11-2(1)*	EAGVAQSPRYKIIEKRQSVAFWCNPI	SGHAT....	LYWYQQILGQGPKLLIQ	FQNNGV..	VDDSQLPKDRFSAERL.KGVDSTLKIQPAKLEDSAVYLC	ASSL.....
21.2	*TRBV11-3*	EAGVVQSPRYKIIEKKQPVAFWCNPI	SGHNT....	LYWYLQNLGQGPELLIR	YENEEA..	VDDSQLPKDRFSAERL.KGVDSTLKIQPAELGDSAVYLC	ASSL.....

8.1	*TRBV12-3*	DAGVIQSPRHEVTEMGQEVTLRCKPI	SGHNS....LFWYRQTMMRGLELLIY	FNNNVP..IDDSGMPEDRFSAKMP.NASFSTLKIQPSEPRDSAVYFC ASSL.....
8.2	*TRBV12-4*	DAGVIQSPRHEVTEMGQEVTLRCKPI	SGHDY....LFWYRQTMMRGLELLIY	FNNNVP..IDDSGMPEDRFSAKMP.NASFSTLKIQPSEPRDSAVYFC ASSL.....
8.3	*TRBV12-5*	DARVTQTPRHKVTEMGQEVTMRCQPI	LGHNT....VFWYRQTMMQGLELLAY	FRNRAP..LDDSGMPKDRFSAEMP.DATLATLKIQPSEPRDSAVYFC ASGL.....
23	*TRBV13*	AAGVIQSPRHLIKEKRETATLKCYPI	PRHDT....VYWYQQGPGQDPQFLIS	FYEKMQ..SDKGSIP.DRFSAQQF.SDYHSELNMSSLELGDSALYFC ASSL.....
16	*TRBV14*	EAGVTQFPSHSVIEKGQTVTLRCDPI	SGHDN....LYWYRRVMGKEIKFLLH	FVKESK..QDESGMPNNRFLAERT.GGTYSTLKVQPAELEDSGVYFC ASSQ.....
24	*TRBV15*	DAMVIQNPRYQVTQFGKPVTLSCSQT	LNHNV....MYWYQQKSSQAPKLLFH	YYDKDF..NNEADTP.DNFQSRRP.NTSFCFLDIRSPGLGDTAMYLC ATSR.....
25	*TRBV16*	GEEVAQTPKHLVRGEGQKAKLYCAPI	KGHSY....VFWYQQVLKNEFKFLIS	FQNENV..FDETGMPKERFSAKCL.PNSPCSLEIQATKLEDSAVYFC ASSQ.....
26	*TRBV17*	EPGVSQTPRHKVTNMGQEVILRCDPS	SGHMF....VHWYRQNLRQEMKLLIS	FQYQNI..AVDSGMPKERFTAERP.NGTSSTLKIHPAEPRDSAVYLY SSG.....
18	*TRBV18(1)*	NAGVMQNPRHLVRRRGQEARLRCSPM	KGHSH....VYWYRQLPEEGLKFMVY	LQKENI..IDESGMPKERFSAEFP.KEGPSILRIQQVVRGDSAAYFC ASSP.....
17	*TRBV19*	DGGITQSPKYLFRKEGQNVTLSCEQN	LNHDA....MYWYRQDPGQGLRLIYY	SQIVND..FQKGDIA.EGYSVSRE.KKESFPLTVTSAQKNPTAFYLC ASSI.....
2	*TRBV20-1*	GAVVSQHPSWVICKSGTSVKIECRSL	DFQATT...MFWYRQFPKQSLMLMAT	SNEGSKA.TYEQGVEKDKFLINHA.SLTLSTLTVTSAHPEDSSFYIC SAR.....
19	*TRBV23-1(1)*	HAKVTQTPGHLVKGKGQKTKMDCTPE	KGHTF....VYWYQQNQNKEFMLLIS	FQNEQV..LQETEMHKKRFSSQCP.KNAPCSLAILSSEPGDTALYLC ASSQ.....
15	*TRBV24-1*	DADVTQTPRNRITKTGKRIMLECSQT	KGHDR....MYWYRQDPGLGLRLIYY	SFDVKD..INKGEIS.DGYSVSRQ.AQAKFSLSLESAIPNQTALYFC ATSDL....
11	*TRBV25-1(1)*	EADIYQTPRYLVIGTGKKITLECSQT	MGHDK....MYWYQQDPGMELHLIHY	SYGVNS..TEKGDLS.SESTVSRI.RTEHFPLTLESARPSHTSQYLC ASSE.....
14	*TRBV27(1)*	EAQVTQNPRYLITVTGKKLTVTCSQN	MNHEY....MSWYRQDPGLGLRQIYY	SMNVEV..TDKGDVP.EGYKVSRK.EKRNFPLILESPSPNQTSLYFC ASSL.....
3	*TRBV28*	DVKVTQSSRYLVKRTGEKVFLECVQD	MDHEN....MFWYRQDPGLGLRLIYF	SYDVKM..KEKGDIP.EGYSVSRE.KKERFSLILESASTNQTSMYLC ASSL.....
4	*TRBV29-1(1)*	SAVISQKPSRDICQRGTSLTIQCQVD	SQVTM....MFWYRQQPGQSLTLIAT	ANQGSEA.TYESGFVIDKFPISRP.NLTFSTLTVSNMSPEDSSIYLC SVE.....
20	*TRBV30(1)*	SQTIHQWPATLVQPVGSPLSLECTVE	GTSNPN...LYWYRQAAGRGLQLLFY	SVGIG...QISSEVP.QNLSASRP.QDRQFILSSKKLLLSDSGFYLC AWS.....

N28 highlighed in red interacts with E94 of SPEA
(TK)55 (DEN)56 K57 highlighted in blue are the sites of SPEA interaction with side chain atoms in CDR2
S49 and R69 highlighted in pink are characterise the CDR2 structure of mV8.2