DETECTION OF BACTERIAL SUPERANTIGENS USING HLA CLASS II

AND

CHARACTERISATION OF THE TcR Vβ SIGNATURE

Miss Lauren Spurling

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Abstract

Bacterial superantigens (SAgs) are protein exotoxins of *Staphylococcus aureus* and *Streptococcus pyogenes* which trigger massive, polyclonal, T cell activation dependent upon the HLA class II molecule, but independent of HLA class II processing. SAgs are responsible for streptococcal and staphylococcal toxic shock syndrome. The staphylococcal SAgs are responsible for enterotoxic food poisoning, whilst SAgs are also hypothesised to have a role in Kawasaki's Disease and other forms of autoimmunity - rheumatoid arthritis, diabetes mellitus, psoriasis and eczema. The investigation, diagnosis and management of SAg mediated disease are hampered by limitations in the tools available for superantigen detection. SAgs are serologically very diverse, are active at tiny concentrations and surrogate markers of superantigenicity, such as Vβ specific changes in T cell repertoire, produce conflicting data even in the definitive syndrome of SAg exposure; toxic shock.

The definitive SAg receptor is the HLA class II molecule and binding affinities vary between ~1nM and ~50μM. SAg binding to HLA class II is centred around a low affinity α chain binding site and a high affinity zinc dependent binding site around a highly conserved histidine residue at position 81 on the β chain. Regardless of serological diversity SAgs bind HLA class II molecules. The fundamental hypothesis behind the work presented here is that this interaction between HLA class II and SAgs could be exploited to develop new approaches to SAg detection.

Affinity columns were generated to purify HLA-DR from HLA homozygous B cell lines. SPEA, C, L and M were expressed in *E. coli* using various vector systems and attempts were made to clone SEJ. Recombinant superantigens were validated by observing Vβ specific T cell responses *in vitro* using flow cytometry. Soluble HLA DR was used to bind a range of bacterial superantigens in an ELISA. Following observations of inter-individual responses to SPEL and SPEM during the expression work, analysis of the Vβ specific responses to superantigens was assessed in HLA typed donors using flow cytometry.

Purified HLA DR binds a range of bacterial superantigens in solution with affinities sufficient to allow detection by ELISA. Zinc chelation using EDTA reduces binding of SPEA, C and M but not SEA to HLA DR.

SPEA expands Vβ14 whilst SPEC targets Vβs 8 and 12. SPEL and M show identical Vβ signatures, expanding Vβs 1, 5.1 and 23. However the hierarchy of response differs between toxins. Marked inter-individual variation in responses to individual superantigens also exists which may be partially explicable by differences in HLA class II. However, despite variations,

the predominant Vβ responding to a particular superantigen remains constant between individuals.

The interaction between HLA class II and superantigens holds the potential be exploited to develop novel superantigen-ligands. Characterisation of Vβ responses to superantigens needs to take account of inter-individual differences in response, superantigen potency and concentration.

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Declaration

I declare that the research contained within this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to these or any other university for a degree, and does not incorporate any material already submitted for a degree.

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Abbreviations

AD	Atopic dermatitis
APC	Antigen presenting cell
ARF	Acute rheumatic fever
ATCC	American tissue culture collection
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CDR	Complementarity determining region
CLIP	Class II associated invariant peptide
CV	Column volume
DMEM	Dulbeccos Modified Eagle Medium
DMP	Dimethyl Pimelimidate
DTT	Dithiothreiol
ECACC	European Collection of Cell Cultures
ELISA	Enzyme Linked ImmunoSorbent Assay
FACS	Fluorescent Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
FR	Framework region
GM	Geometric mean
GST	Glutathione S transferase
HLA	Human Leukocyte Antigen
HRP	Horseradish peroxidase
HV	Hypervariable region
Ii	Invariant chain
IMGT	International ImMunoGeneTics Project
IPTG	Isopropylthio-β-D-galatosidase
KD	Kawasaki's Disease
LB	Luria Bertani
FCS	Fetal calf serum
MAM	*Mycoplasma arthritidis* associated mitogen
MHC	Major histocompatibility
MMTV	Mouse mammary tumour virus
P_{50}	Half maximal proliferation
PBMCs	Peripheral blood monocyte cells
PBS	Phosphate buffered saline solution
PE	Phycoerythrin

PpL	Protein L
PrtA	Protein A
PrtG	Protein G
RA	Rheumatoid arthritis
RT	Room temperature
SAg	Superantigen
SDM	*Streptococcus dysgalactiae* derived mitogen
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE (A)	Staphylococcal enterotoxin (A)
SMEZ	Streptococcal mitogenic exotoxin Z
SPE (A)	Streptococcal pyrogenic exotoxin (A)
SPR	Surface plasmon resonance
SSA	Streptococcal superantigen
SSL	Staphylococcal superantigen like protein
sTSS	Streptococcal toxic shock syndrome
TcR	T cell receptor
TMB	Tetramethyl benizidine
Trx	Thioredoxin
TSB	Tryptone soya broth
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin 1
Vβ	Variable beta region
YPM-(A)	*Yersinia pseudotuberculosis* mitogen (A)

1. Introduction

1.1 – Background

1.1.1 – General considerations

In the late 1960s a team of researchers led by Bergdoll isolated a potent secreted enterotoxin, Staphylococcal enterotoxin A (SEA), from *Staphylococcus aureus* [1]. Marrack and Kappler, working on the same toxin in 1990, discovered that SEA also had a potent mitogenic effect on T cells, and was capable of stimulating a very rapid and polyclonal proliferation and expansion of T cells with specific Vβ regions [2]. This bore a striking similarity to the previously identified minor lymphocyte stimulating (Mls) in mice. These Mls antigens stimulated murine T cells bearing particular Vβs [3, 4] and were later found to be encoded within the mouse mammary tumour virus (MMTV) genome [5]. With this discovery, Marrack and Kappler termed the family of bacterial toxins isolated since Bergdoll's original discovery as 'superantigens', to account for their potent mitogenicity and antigenicity [2, 6].

Since the initial discovery and subsequent classification of SEA, many other superantigens (SAgs) have been isolated from *S. aureus* and *Streptococcus pyogenes.* The classical bacterial superantigens of *S. aureus* and *S. pyogenes* are protein exotoxins of between 20-30kDa in size [7]. By activating T cells on the basis of their Vβ type they cause massive polyclonal T cell activation and production of inflammatory cytokines [8]. It is this affect which is thought to be behind their involvement in human diseases such as toxic shock syndrome (TSS), Kawasaki's Disease (KD), acute rheumatic fever (ARF) and autoimmune disorders such as atopic dermatitis [9, 10]. Importantly, they elicit these effects by binding the HLA class II molecule and T cell receptor simultaneously, at sites distant from the peptide specific regions [8]. It was originally believed that only some strains of *S. aureus* and *S. pyogenes* were toxigenic, however recent genomic evidence indicates that almost all strains carry multiple superantigen genes [8, 11, 12]. The work described in this thesis focuses mainly on the streptococcal superantigens, however staphylococcal superantigens are also considered.

1.1.2 Superantigen Structure

Crystal structures for staphylococcal SAgs SEA,[13] SEB [14], SEC2 and 3 [15, 16], SED [17], SEG [18], SEH [19], SEI [20], SEK [21] and TSST-1 [22], and streptococcal SAgs SPEA [23], SPEC [24], SPEH [25], SPEI [26], SPEJ [27] and SMEZ2 [25] have been published in recent years. These structures have revealed a highly conserved tertiary structure between SAgs,

despite sequence homology varying between 5% and 95% [26]. Mature toxins consist of two globular domains – an N-terminal β-barrel domain and a C-terminal β-grasp domain, joined by an α-helix [10]. The β-barrel domain, sometimes referred to as the OB oligonucleotide fold consists of a 5 stranded β sheet, folded to form a barrel shape. This is a structure found widely throughout bacterial species, in particular in cholera toxin and verotoxin in *E. coli* [28]. The β-grasp domain, so named due to the appearance of a β-sheet 'grasping' the central α helix [28], is found in many binding proteins and protein modification molecules, including ubiquitin, bacterial ferrodoxins and streptococcal immunoglobulin binding proteins Protein L and Protein G [29]. That the two domains of superantigens so closely resemble domains present in many other bacterial proteins adds weight to the hypothesis that SAgs probably evolved as a result of the combination of two other toxins [29]. The toxins are very soluble, heat resistant, protease resistant and non-glycosylated [30, 31].

All superantigens possess at least one HLA class II binding site. The first of these HLA class II binding sites is located in the N-terminal β barrel domain. This site binds HLA class II with low affinity. The second HLA class II binding site is in the C-terminal β grasp domain and binds HLA class II zinc dependently with high affinity [32, 33]. All SAgs also possess a TcR binding domain, usually located within the β-barrel domain [7]. Superantigens are produced as pre-cursor molecules, with an N-terminal signal peptide, which is linearised at the bacterial cell surface to enable secretion [31]. Figure 1.1 below shows a ribbon structure of a typical superantigen, SEB.

Figure 1.1 Generic structure of superantigens.

β sheets are shown in green, α helices in red and loops in yellow. The various domains and HLA class II and TCR binding sites are labelled. Diagram adapted from the structure of SEB as depicted in Papageorgiou *et al* 1998[33] and Figure 1.2, Llewelyn 2004 [34].

1.2 The Human Leukocyte Antigen class II complex

1.2.1 General Considerations

The MHC class II complex, referred to in human immunology as HLA class II, is an important immunological receptor expressed by antigen presenting cells (APCs) [35]. This molecule presents peptide antigen on the surface of APCs to the T cell receptor on CD4 positive T cells [36], eliciting T cell proliferation and cytokine induction, as well as recruitment of B cells and the subsequent production of immunoglobulin [35]. The receptor is a 60kDa glycoprotein heterodimer formed of two transmembrane subunits, α and β (33-35kDa and 25-30kDa respectively) [36, 37] (Figure 1.2). Each subunit contains two domains, α2 and α2, β1 and β2 joined by a short transmembrane region. Domains α2 and β2 closely resemble the constant regions of immunoglobulin, whilst domains α1 and β1 form an open ended peptide binding groove which is specific for a particular peptide antigen [36]. Generally the HLA class II molecule presents exogenous peptide, that is to say peptide from proteins outside the cellular environment which the APC has processed. This may be host or foreign peptide. [36].

Three isotypes of HLA class II exist in humans, HLA-DR, DP and DQ, with DR the most strongly expressed [36]. HLA class II is expressed constitutively on antigen presenting cells including B cells, macrophages and dendritic cells to a lower level. Expression can be induced by interferon gamma (IFNγ) on other cells such as thymic T cells and epithelial cells [38]. Two isotypes exist in mice, known as I-A and I-E [39]. HLA-DM is a molecule which assists in the peptide loading of HLA-DR, DP or DQ and has no role in peptide recognition [38]. HLA class II α and β subunits are synthesised separately with N-terminal signal peptides which initiate their translocation to the endoplasmic reticulum [36]. Here the two subunits are folded with the assistance of chaperone proteins including invariant chain (Ii) [40]. Ii chain forms a trimer in its native state, and therefore associates with three αβ HLA class II heterodimers, which may be any combination of DR, DQ and DP alleles [36]. Ii chain prevents the binding of immunogenic peptide, but acts to stabilise class II structure. When peptide antigen is ready to be loaded into class II, Ii is linearised by proteolysis to a smaller fragment known as class II associated invariant peptide (CLIP). HLA-DM, which requires no stabilising chaperone proteins, transfers peptide into the peptide binding groove of HLA class II, removing the CLIP fragment in the process [41]. Peptide loaded HLA class II is then transported to the cell surface with the help of additional chaperone proteins. The receptor is then expressed on the cell surface [42, 43]. It is important to consider the intracellular pathway of peptide processing and antigen presentation as although SAg-HLA class II interactions are extracellular, some SAgs make extensive contacts with peptide antigen [44, 45].

1.2.2 HLA class II nomenclature

The human HLA class II genes are located within the MHC complex region in the short arm of chromosome 6 [46]. No current standardised nomenclature currently exists for gene products within this region; therefore nomenclature described here refers to the genes themselves. The HLA class II genes are among the most polymorphic in the human genome, contributing to very diverse antigen specificity, however this also means that currently more than 1500 alleles within this region have been sequenced [46]. Fortunately alignments of these alleles can now be viewed as nucleotide and amino acid sequence using the IMGT/HLA database [39].

This research focuses mainly on HLA-DR1 β chains, therefore nomenclature for other HLA class II elements will not be discussed here. HLA-DR is composed of a monomorphic α chain, coded for by alleles named *DRA1*. Four possible DR β chains exist, *DRB1, DRB3, DRB4* and *DRB5*. 3 DR α chain alleles and 707 DR β chain alleles have been sequenced. This includes allelic variants and polymorphisms [39, 47]. To explain the nomenclature using the example *DRB1*04030, DRB1* refers to the gene of interest, *04* is the allele family, often corresponding to serotype although sometimes these numbers differ (Table 1.1), *03* refers to the order which sequences were described and *01* refers to polymorphisms within that allelic family [39, 47]. The HLA-DR β chain alleles used in this work and their corresponding serotypes are summarised in Table 1.2.

Table 1.1 HLA types referred to in this research

Serotypes taken from IMGT/HLA database [39]

HLA β chain allele	**Serotype**
*DRB1*04(03)*	DR4
*DRB1*0701*	DR7
*DRB1*1101*	DR5
*DRB1*1301*	DR6

1.2.3 Superantigen interactions with HLA class II

Eight superantigen HLA class II complexes have been crystallised and the details of the binding interaction published [20, 32, 45, 48-52]. Together with *in vitro* mutagenesis studies, these have revealed two distinct binding sites on the HLA class II molecule away from the peptide groove (Figure 1.2), as well as a number of different binding modes.

1.2.3a Interactions with HLA class II α chain

One SAg binding site has been identified on each chain of the molecule. The binding site on the monomorphic DR α-chain is a low affinity site. SAgs binding here have a dissociation constant (K_D) of around 10^{-5} M [7, 53]. Crystallisation studies have shown that binding of SAgs such as SEB and TSST-1, which only possess a site for low affinity α chain binding, occurs via a hydrophobic interaction [54]. The hydrophobic core of the SAg N-terminal domain, in the form of a ridge, interacts with hydrophobic residues present in a groove in the α1 region of HLA class II α chain [55]. In particular the hydrophobic residues Y13, M36, A37, L60, I63 and A64 seem to be important in this hydrophobic groove, although these residues may differ between SAgs [7, 48, 53, 56]. More recently αLys39 has been identified as a highly conserved residue within the hydrophobic groove of both HLA-DR and DQ and appears to be an important site in SAg binding, forming a salt bridge between HLA class II and residues present in the hydrophobic ridge of SAgs [23, 55, 57]. Whilst the low affinity binding site on HLA class II is the same for all SAgs which use this site, the toxins bind in very different ways. SEB for example binds out to the side of the HLA molecule, away from the peptide binding groove [54], whilst TSST-1 binds across the peptide binding groove and interacts with the peptide antigen [7, 49, 58].

1.2.3b Interactions with HLA class II β chain

The second site identified as a SAg binding site is found on the highly polymorphic β chain of HLA class II. SAgs bind here with high affinity, with a K_D of around 10^{-7}M – around 100 times higher affinity than for the low affinity site [55]. This site contains a highly conserved histidine residue, H81 which appears to be crucially important in the binding of SAgs [7, 31, 45, 59]. High affinity binding is zinc mediated [16, 32, 45, 59-63]. The zinc cation and three residues in the relevant SAg's C-terminal domain form a tetrameric complex, which then associates with H81 in the HLA class II β chain [7, 45, 58, 62-64]. In addition to the conserved H81 residue on the β chain, several sites have been identified as important in high affinity binding from crystal studies. These sites usually make hydrogen bonds or van der Waals interactions with SAg residues. Hudson *et al* first demonstrated that mutations in residues R71, R72, A74, D76, T77, R80, Y83, G84 and V85 concurrently reduced binding of SEA to HLA-DR1, but that mutations in residues C79, R80, Y83 and V85 had no effect on binding [64]. Petersson *et al* then identified Thr77, Asp76 and His81 as important residues for SEH binding to HLA-DR1, followed by Li *et al* who demonstrated hydrogen bonds or van der Waals forces between SPEC and Asp70, Ala73, Asp76, Thr77, Arg80, His81 and Gly84 [32, 45]. Finally Fernandez identified residue interactions between SEI and Glu69, Asp76, Thr77, Arg80 and His81 [20]. Mutations in these sites may reduce the capability to form hydrogen bonds or van der Waals interactions with SAg residues due to changes in hydrophobicity or charge. Also, residues at these sites are not conserved and differ between HLA class II alleles (Table 1.2).

Polymorphisms at these sites may contribute to variations in superantigen responses observed between HLA types [65-67].

Table 1.2 Important amino acids positions for SAg binding on HLA-DR β chain

HLA alleles shown are commonly expressed types [68]. H81 is well known to be conserved in HLA-DR [64]. Other important positions have been identified using crystallographic studies of SAg/HLA class II interactions. Important sites identified in *DRB1*0401* and *DRB1*0201* product [20, 32, 45, 64, 69]. Differing amino acids are highlighted in yellow.

*DRB1**	69	70	76	77	80	81	84
0101	E	Q	D	T	R	H	G
0401	E	K	D	T	R	H	G
1101	E	D	D	T	R	H	G
1501	E	Q	D	T	R	H	G

Some SAgs, for example SEA and SEE possess a low affinity binding site and a high affinity binding site, allowing them to bind to both sites on HLA class II and crosslink HLA molecules by bringing them closer together within lipid rafts [31]. As is often the case with immunological receptors, this cross-linking appears to have a crucial role in the expression of genes encoding inflammatory cytokines [70] within antigen presenting cells such as monocytes [31]. Other SAgs, for example SMEZ and SPEH, only bind the high affinity β chain site, and are known to only contain one HLA binding site [19, 31, 71], whilst other SAgs (SPEC for example) are known to dimerise and therefore can also cross link two HLA class II molecules [71]. SEH binds across the top of the peptide binding groove of HLA class II, utilising the high affinity β chain binding site, and demonstrates the highest affinity for HLA class II molecules in any known SAg (1nM) [45, 72].

1.2.3c Classification of superantigens by HLA class II binding mode

Various binding modes of SAg to HLA class II exist. SAgs possessing only the low affinity binding site (Tables 1.3a+b) bind class II via α chain only [54]. SAgs possessing both low and high affinity binding sites (Tables 1.3a+b) can crosslink two HLA class II molecules by binding both α and β chain sites [31], exaggerating expression of inflammatory cytokines [70] within APCs [31]. HLA class II alleles are very polymorphic and this may affect the affinity of superantigens for the high affinity binding site. This may consequentially have an impact on an individual's susceptibility to superantigen mediated disease [66, 73]. Additionally although

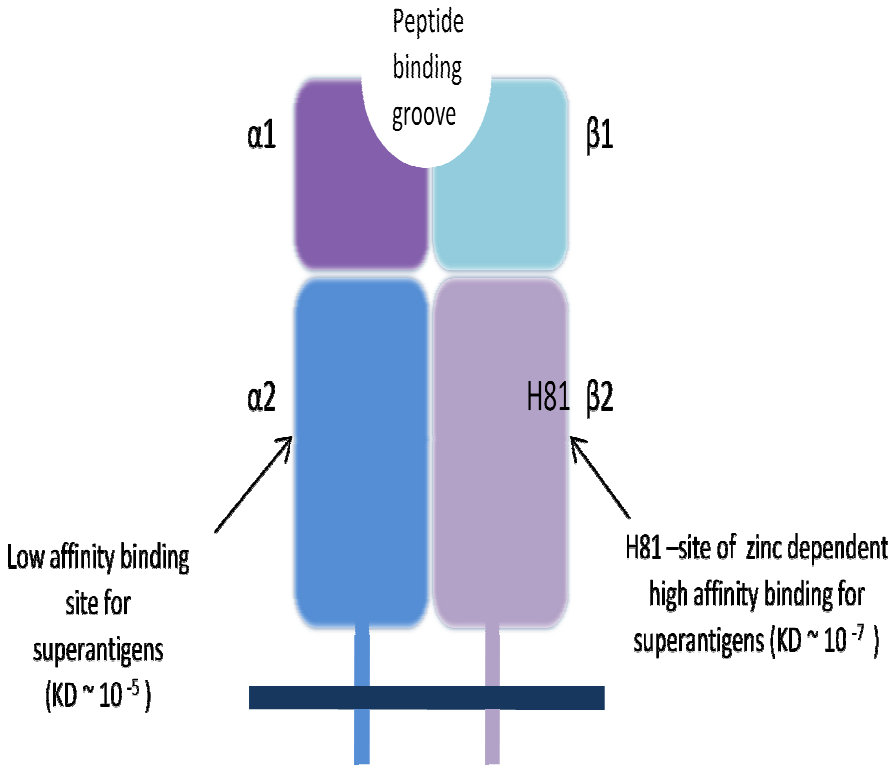
most SAgs bind HLA-DR, some (e.g. SSA and SPEA) preferentially bind HLA-DQ [65-67]. Some SAgs only bind the high affinity β chain site, and only contain one HLA binding site [31], whilst other SAgs also interact with peptide antigen (TSST-1) [22]

As the sequence homology of superantigens varies so greatly, it has long been a challenge to group the superantigen toxins into families. The latest literature classes the superantigens into five families based on structure, functionality, evolutionary phylogeny and sequence homology, in addition to their HLA class II binding modes. These five groups are named Groups I-V and can be summarised as follows:

- ***Group I*** – this group contains only one toxin, TSST-1, the toxin responsible for most cases of menstrual toxic shock syndrome. TSST-1 possesses many unique qualities and shares very little homology with any other superantigen [26].
- ***Group II*** – these superantigens, including SEC1, 2, and 3, SEA, SSA and SPEA bind HLA class II through the low affinity class II binding site in a peptide independent manner. The Vβ element of the TcR is bound through conformational dependent mechanisms, and the individual Vβ side chains have very little effect on binding [26].
- ***Group III*** – group III SAgs are largely restricted to staphylococcal SAgs, with the exception of SPEH and SePEH, and are the agents most commonly associated with food poisoning. These SAgs contain binding sites for the low affinity class II site and the high affinity zinc dependent class II site, and can therefore crosslink class II molecules. Little is known about the Vβ interactions which occur here [26].
- ***Group IV*** – only streptococcal SAgs are present in this group with the exception of SET, recently identified by Ono *et al* [74]. These SAgs bind HLA class II via the high affinity zinc dependent binding site and some, for example SPEC, are hypothesised to not contain a low affinity site [24, 63]. Engagement of Vβ elements is very distinctive with these SAgs, involving multiple side and main chain atoms of all three CDRs, HV4 and FR3 [75, 26].
- ***Group V*** – this group includes SPEI and SEI, K, L, M and Q. It has been shown with SEI that the β-grasp domain interacts with the α1 chain of HLA-class II, as well as with the N-terminal of the antigenic peptide present in the antigen binding groove of HLA class II [20]. This resembles a structure similar to that of Group IV SAg SPEC and HLA-DR, and Group III SAg SEH with HLA-DR [26]. However Group V SAgs also possess a unique loop between α helix 3 and β sheet 8 in the β-grasp domain, the functionality of which has not yet been established [26]. Figure 1.3 shows the phylogenetic similarity between known superantigens.

Figure 1.2 Superantigen binding sites on

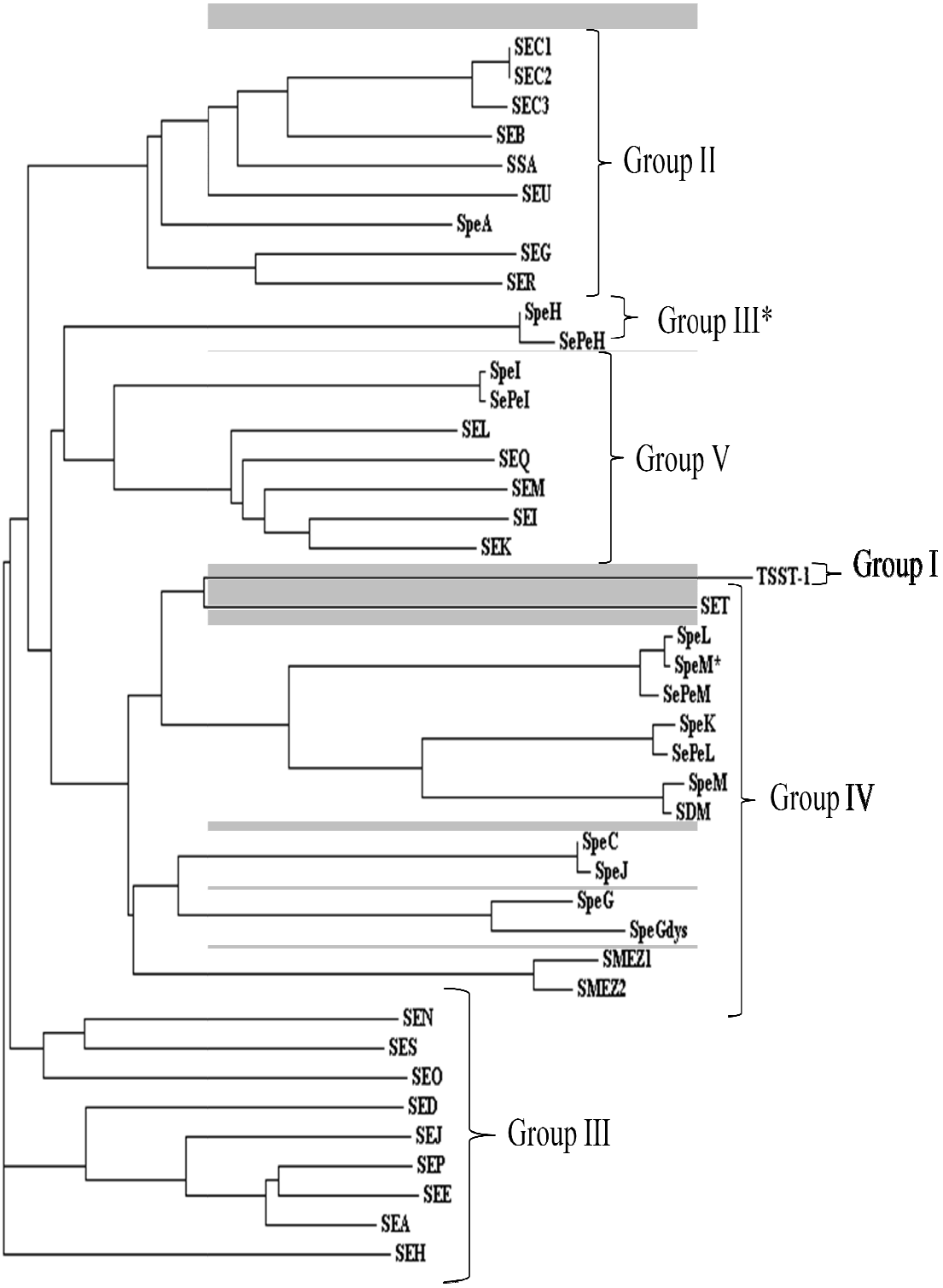
Showing α and β chains of HLA class II, the peptide binding groove, and distinct binding sites on both chains of the molecule. Diagram adapted from Immunobiology 6



Adapted from fig 3.21b p123 Immunobiology, Janeway et al

Figure 1.3 Phylogenetic similarity of superantigens

A phylogenetic analysis of the staphylococcal and streptococcal superantigens generated using Figure created from accession numbers from Brouillard 2007 [26] * Using structural data from [26] SpeH and SePeH also belong in Group III



1.3 Superantigen interactions with the T cell receptor

1.3.1 General considerations

The T cell receptor (TcR) is the receptor present on all CD4 and CD8 T-cells. It is a heterodimer formed of two glycoprotein polypeptide chains, either an α and β chain or a γ and δ chain. The αβ TcR is by far the most widely expressed [76]. Antigen presenting cells of the immune system present peptide antigen to T cells, which if recognised through the TcR peptide binding region and CD4 or CD8 elicits appropriate intracellular pathways and therefore T cell responses [38]. Conventional peptide antigen stimulation of T cells thus leads to activation of 1:1000 to 1:10000 T cells [77].

Each chain of the TcR is formed of variable (V), junction (J) and constant (C) regions. The β chain also contains diversity (D) regions [35] (Figure 1.4). Each of these regions is expressed by a separate gene located on non-contiguous regions of chromosome 7 for β chain and chromosome 14 for α chain in humans [78]. The α and β genes are highly homologous [79]. Framework regions (FR) interspersed throughout the molecule help to maintain structural stability [80]. Somatic rearrangement of V, D and J (β and δ chains) or V and J (α and γ chains) gene segments during cell division gives rise to a very wide antigenic diversity [38, 81].

C regions of the T cell receptor bear a striking sequence homology to the constant regions of immunoglobulin whilst the V regions contain hypervariable sequences (HV regions) which are equivalent to the complementarity determining regions of immunoglobulin (CDRs), and confer specificity [38, 76]. These regions are often referred to as CDR regions 1-3 due to their similarity to the immunoglobulin CDR regions [80]. The β chain also contains a fourth hypervariable domain (HV4) which appears to be involved in superantigen binding [26]. Most superantigens bind the Vβ region in some way [58].

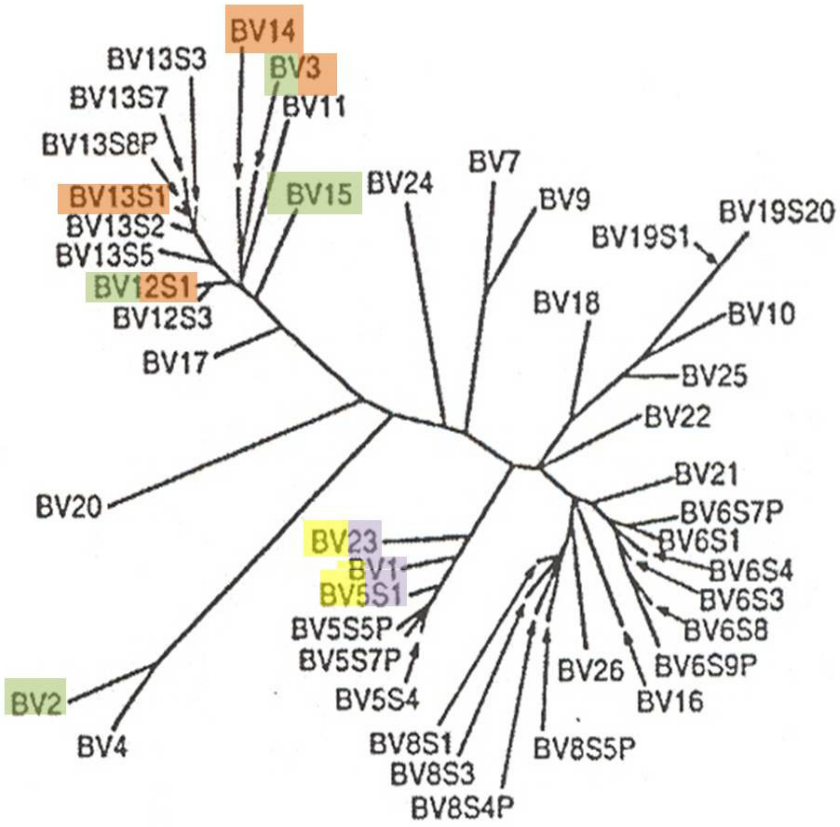
Currently 47 functional Vβ and 42 functional Vα gene segments are described [79]. The Vβ and Vα genes, referred to genetically as *TCRBV* and *TCRAV* respectively are classified into subfamilies based on 75% identity at the nucleotide level. These are referred to as *TCRAV1, TCRBV2* etc [82]. Comparison of gene sequences has allowed phylogenetic trees showing relationship of Vα and Vβ genes to be created. Figure 1.5 shows a phylogenetic tree for the Vβ genes with certain Vβ subtypes studied in this research highlighted.

Figure 1.4 T cell receptor

Showing a generalised structure of an αβ T cell receptor. Superantigens generally bind the Vβ region. Diagram adapted from Fig 3.1 p 116 Immunobiology 6th edition, and Figure 1.3 Llewelyn 2004 [34, 35].

Figure 1.5 Human TCRBV families

Phylogenetic similarity between members of the TCR Vβ gene family. Figure adapted from Arden *et al* 1995 [79] and Figure 5.22 Llewelyn 2004 [34]. Vβs documented to respond to SPEA, SPEC, SPEL and SPEM are highlighted in orange, green, yellow and purple respectively [58, 66, 73].



1.3.2 Superantigen interactions with the T cell receptor

Most SAgs bind the Vβ region of the TcR [72, 83] and most will bind more than one Vβ type. As the number of major Vβ types (e.g. Vβ1, Vβ2 etc) in humans is limited to around 26, comprising 47 functional Vβ chains (e.g. Vβ5.1, Vβ5.2. Vβ5.5 etc) [79] [7], and each SAg will bind more than one, the number of T cells which can be activated by superantigens is up to 20% of the hosts repertoire [8, 44]. The Vβs which a superantigen expands are referred to as the Vβ repertoire or signature. It should be noted that Vβ specific responses to SAgs are species specific [8] so that in the case of TSST-1 for example TcR Vβs 3, 10, 15 and 17 respond in mice, but in humans responses in Vβ2 are seen [2]. TcR-SAg binding is less well characterised than HLA class II-SAg binding, probably due to the wider range of TcR Vβ regions compared to HLA class II isotypes. However 5 TcR-SAg complexes have been crystallised [15, 18, 51, 84, 85]. From these it has been inferred that binding involves sites in the CDR2, HV4, FR2 and FR3 of the Vβ region, although not all these regions may be involved in the binding of all superantigens. These regions are believed to interact with the α-helix region of SAg, between the N and C terminal domains [86]. Binding to the TcR also involves interaction with the CDR3 region of Vβ, and SAgs usually stimulate Vβs with similar sequences in this region [26, 79]. In particular HV4 seems to be important in binding, especially for some streptococcal superantigens possessing a novel loop between the two active domains of the toxin [26]. The Vβ specific response has been described as almost all T cells of a specific Vβ responding [2]. Cells of a particular Vβ may not respond due to the peptide dependence of some SAg binding modes [45]. The specific binding requirements of some superantigens, for example MAM which utilises a very specific Jβ region in its binding, may also cause a small subset of T cells bearing a particular Vβ type not to respond [87]. There are also sub-populations of T cells such as natural regulatory T cells (nTregs) which although may be of the correct Vβ, do not proliferate in response to superantigen stimulation, but act in a regulatory capacity [21]. Vα involvement with SAgs (SEH, [57]) may also play a role here.

1.4 The microbial superantigens

Superantigens have now been described in a number of bacteria and viruses. This section will describe the classical superantigens of *Staphylococcus aureus* and *Streptococcus pyogenes* before describing SAgs in other bacterial species, as well as endogenous and viral SAgs.

1.4.1 Bacterial Superantigens

1.4.1a Staphylococcus aureus

Since the initial isolation of SEA by Bergdoll and co-workers, a total of 12 staphylococcal SAgs have been identified – staphylococcal enterotoxin (SE) A, B, C1-3, D, E, G-U and toxic shock syndrome toxin 1 (TSST-1) [26, 58, 74, 88]. These toxins range from between 21.9 kDa (TSST-1) and 28.5 kDa (SEJ) in size [7]. Most of these are potent emetic agents. [89, 90]. The staphylococcal enterotoxins and TSST-1 are also pyrogenic, inducing high fevers in the infected host [90]. TSST-1 is unique in that it can cross the mucosal surfaces. Most strains of *S. aureus* carry genes for more than one of the toxins, suggesting a high occurrence of horizontal transfer of genetic material between strains [91]. The staphylococcal SAgs studied thus far vary considerably in their potencies. For example, SEA demonstrates half maximal proliferation of T cells (P_{50}) at 0.1pg/ml, whilst SEB has a P_{50} of 0.8pg/ml [7]. Dissociation constants for HLA class II and SEA, B, C3, H and TSST-1 have been calculated using surface plasmon resonance (SPR) and are 9-15nM, 14µM, 48µM, 1nM and 100nM respectively [7, 45, 64, 86]

Of all the staphylococcal SAgs, only SEG, H and I are found in the chromosomal genome, and even these are likely to have arisen as a result of genetic transfer and integration of genes from other sources such as phages [91]. Other staphylococcal SAgs have long been known to be associated with mobile genetic elements such as prophages (SEA, SEE and SEP), pathogenicity islands, either chromosomal or plasmid (SEB, SEC, SEG, SEI, SEM, SEN, SEO, SEK, SEL, SEQ, TSST-1) or plasmids (for example SED and SEJ are found on plasmid pIB485). Furthermore, recombination within pathogenicity islands gives rise to novel superantigens such as SEU and SET [74, 91].

1.4.1b Streptococcus pyogenes

At present 12 streptococcal pyrogenic exotoxins (SPEs) have been described in the literature – SPEA, SPEC, SPEG, SPEH, SPEI, SPEJ, SPEK, SPEK/L, SPEL/M, SPEM* SSA and SMEZ1 and 2 [31, 58]. SPEB, originally designated as a superantigen, has since been shown to be a potent cysteine protease and may be important in the regulation of other SAgs including SMEZ1 and 2 [92]. Original purifications of SPEB showing mitogenic activity were probably contaminated with very low concentrations of the very potent SAg SMEZ2 [58]. The

streptococcal superantigens vary in size between 23.6 kDa (SPE-H) and 27.4 kDa (SPEK/L) [7]. SPEA and SPEC are the causative agents in diseases such as scarlet fever, and SPEA is also strongly epidemiologically linked to streptococcal toxic shock syndrome (STSS), which bears a striking resemblance to toxic shock syndrome caused by *S. aureus*, also strongly linked to superantigens such as TSST-1 and SEA, including an erythematous blanching rash, hypotension and multi-organ failure [31]. The streptococcal superantigens are not emetic but are also pyrogenic [31]. SMEZ1 and 2 (Streptococcal mitogenic exotoxin Z) are allelic variants, of which there are at least a further 31, and are by far the most potent superantigen identified thus far [93]. SMEZ1 and SMEZ2 show P_{50}s of 0.08pg/ml and 0.02pg/ml respectively [7]. SPEA also exists in at least 4 allelic variants [94]. Other streptococcal SAgs studied show P_{50}s ranging from 0.1pg/ml (SPEC, SPEI, SPEJ) to 50pg/ml (SPEH) [7].

Similarly to staphylococcal SAgs, the genes encoding most streptococcal SAgs originate from phages or are located on other mobile genetic elements, therefore the toxins produced varies considerably between strains. The genes encoding SPEG and SMEZ however are chromosomal and are found in more than 90% of *S. pyogenes* strains [31].

Tables 1.3a and 1.3b summarise the known properties of classical superantigens.

Table 1.3a Properties of the Staphylococcal Superantigens

Information collated from Proft *et al* 2003 and 2008, Hudson and Li. [7, 58, 64, 86]

SAg	Mwt (kDa)	Amino Acid (mature protein)	Gene Location	Class II binding	Zinc binding	MHC affinity	TCR affinity	P50 (pg/ml)	Human TCR Vβ-Specificity
Staphylococcal Superantigens									
SEA	27.1	233	Phage	α/β (DR) peptide	Y	9-15nM		0.1	1.1, 5.3, 6.3, 6.4, 6.9, 7.3, 7.4, 9.1, 23.1
SEB	28.4	238	Phage	α/- (DR)	N	14μM	140μM	0.8	1.1, 3.2, 6.4, 15.1
SEC1	27.5	239	Phage	α/- (DR)	N		0.9μM	0.2	3.2, 6.4, 6.9, 12, 15.1
SEC2	27.6	239	Phage	α/- (DR)	N			0.2	12, 13, 14, 15, 17, 20
SEC3	27.6	239	Phage	α/- (DR)	N	48μM	4.5μM	0.2	5.1, 12
SED	26.9	233	Phage	α/β (DR)	Y				1.1, 5.3, 6.9, 7.4, 8.1, 12.1
SEE	26.8	228	Phage	α/β (DR)	Y			0.2	5.1, 6.3, 6.4, 6.9, 8.1
SEG	27.0	233	Chromo	?	?				3, 12, 13.1, 13.2, 14, 15
SEH	25.2	217	Chromo	-/β (DR) peptide	Y	1nM			None – binds to Vα
SEI	24.9	218	Chromo	?	?				1.1, 5.1, 5.3, 23
SEJ	28.5	242	Phage	?	?				?
SEK	25.3	219	Phage	?	?				5.1, 5.2, 6.7
SEL	24.7	216	Phage	?	?				?
SEM	24.8	214	Phage	?	?				?
SEN	26.1	?	Phage	?	?				?
SEO	26.7	?	Phage	?	?				?
SEP	26.4	?	Phage	?	?				?
SEQ	26.0	?	Phage	?	?			0.2	2.1, 5.1, 21.3
SE/U	?	?	Phage	?	?	?	?	?	13.2, 14
SE/V	?	?	Phage	?	?	?	?	?	6, 18, 21
TSST	21.9	194	Phage	α/- (DR) peptide	N	100nM			2.1

Table 1.3b Properties of Streptococcal Superantigens

Information collated from Proft *et al* 2003 and 2008, Hudson and Li. [7, 58, 64, 86]

SAg	**Mwt (kDa)**	**Amino Acid (mature protein)**	**Gene Location**	**Class II binding**	**Zinc binding**	**MHC affinity**	**TCR affinity**	**P50 (pg/ml)**	**Human TCR Vβ-Specificity**
Streptococcal Superantigens									
SPE-A	26.0	221	Phage	α/- (DQ)	Y	104nM	6.2μM		2.1, 12.2, 14.1, 15.1
SPE-C	24.4	207	Phage	-/β (DR)	Y			0.1	2.1, 3.2, 12.5, 15.1
SPE-G	24.6	210	Chromo	-/β	Y			2	2.1, 4.1, 6.9, 9.1, 12.3
SPE-H	23.6	204	Phage	-/β (DR)	Y			50	2.1, 7.3, 9.1, 23.1
SPE-I	26.0	227	Phage	-/β	Y			0.1	6.9, 9.1, 18.1, 22
SPE-J	24.6	208	Phage	-/β	Y			0.1	2.1
SPE-L/K	27.4	227	Phage	-/β	Y			1	1.1, 5.1, 23.1
SPE-L/M	26.2	211	Phage	-/β	Y			10	1.1, 5.1, 23.1
SPEM*	25.3	-	Phage	?	Y				1.1, 5.1, 23.1
SSA	26.9	234	Phage	(DR)	N				1.1, 3, 15
SMEZ1	24.3	209	Chromo	?/β (DR)	Y			0.08	2.1, 4.1, 7.3, 8.1
SMEZ2	24.1	209	Chromo	?/β (DR)	Y			0.02	4.1, 8.1

1.4.1c Other Bacterial superantigens

In addition to the toxins found in *S. aureus* and *S. pyogenes,* toxins with similar properties and causing similar disease in animals have been described in various other bacterial species.

Closely related bacteria

Four SAgs have been isolated from *Streptococcus equi* (SePE-H, I, L and M) [7]. *S. equi* is a pathogen of horses and causes various infections. Among these is strangles, a infection of the upper respiratory tract which shares many of the typical characteristic symptoms of toxic shock syndrome, including high fever and an erythematous rash [95]. Generally the clinical spectrum of *S. equi* is very similar to that described for *S. pyogenes* [96]. Genetic analysis of the *S. equi* toxins reveals that they are homologous to their *S pyogenes* equivalents and probably arose as a result of genetic crossover [97]. Three SAgs have thus far been identified from *Streptococcus dysgalactiae* (*S. dysgalactiae*-derived mitogen SDM, SPEA7 and $SPEG^{dys}$) [7]. SDM and $SPEG^{dys}$ appear to be orthologous to SPEM and SPEG respectively, although they share approximately only 30% sequence homology with their orthologues [7]. Other close relatives of *S. pyogenes* exist, including *Streptococcus canis* and *Streptococcus suis* which cause similar diseases to *S. pyogenes* in domestic animals, for example *S. canis* has been shown to be the cause of canine toxic shock syndrome and some cases of canine necrotising fasciitis, however the production of superantigens by these organisms remains to be elucidated [98, 99].

Distantly related bacteria

So far only two superantigens have been identified in Gram negative bacteria. These are *Yersinia pseudotuberculosis*-derived mitogen A and B. Whilst YPM-A and B are superantigenic in the classic definition, promoting expansion of Vβ specific T cells, structurally they are quite different to all other superantigens identified. They are much smaller than other SAgs at 14.5 and 14.6 kDa respectively and do not appear to have the same conserved tertiary structure found in other superantigens [7, 100, 101].

A final superantigen is produced by *Mycoplasma arthritidis (Mycoplasma arthritidis*-derived mitogen). MAM binds preferentially to HLA-DR and targets T cells bearing Vβ6 and Vβ8, quite a different profile to any of the 'classic' SAgs. Recently the ternary HLA class II-MAM-TcR structure was solved and has revealed that MAM bears little structural resemblance to the classical SAgs, but it has been hypothesised that MAM also engages the CDR3 region of the TcR, in addition to the Vβ regions mentioned [102, 7, 52].

The staphylococcal superantigen-like toxins

Although SAgs possess widely diverse sequence homology, two conserved motifs have been identified using the PPSearch tool on the ExPaSy/PROSITE website [103]. These are detailed

in Table 1.4 below. These conserved sequences form the basis for the interface between the N-terminal β barrel and C-terminal β-grasp domain, but have little implications for HLA class II or TcR binding sites [58]. Scanning bacterial genomes for these conserved motifs has identified a separate group of toxigenic proteins in *S. aureus* called the staphylococcal superantigen like toxins (SSLs), designated SSL1-14 [58]. The SSLs are sometimes referred to as staphylococcal enterotoxin like proteins or SEls [104].

These bear a structural resemblance to conventional SAgs, in particular TSST-1 [58, 105]. The genes for these toxins are found within pathogenicity island SaPIn2 of *S. aureus.* Although the SSLs are closely structurally related to other SAgs, they do not have a mitogenic effect on T cells and appear to target molecules of the innate immune system instead [58, 105].

Table 1.5 Conserved sequences of SAgs

Information derived from ExPaSy PROSITE and Proft 2008 [58, 106]

PROSITE Number	**Motif**
PS00277	- G(2) - (LIV) - T–(I)–(N) - x(2) - N
PS00278	K - x(2) - (LIVF) - x(4) - (LIVF) - D - x(3) - R - x(2) - L - x(5) - (LIV) - Y

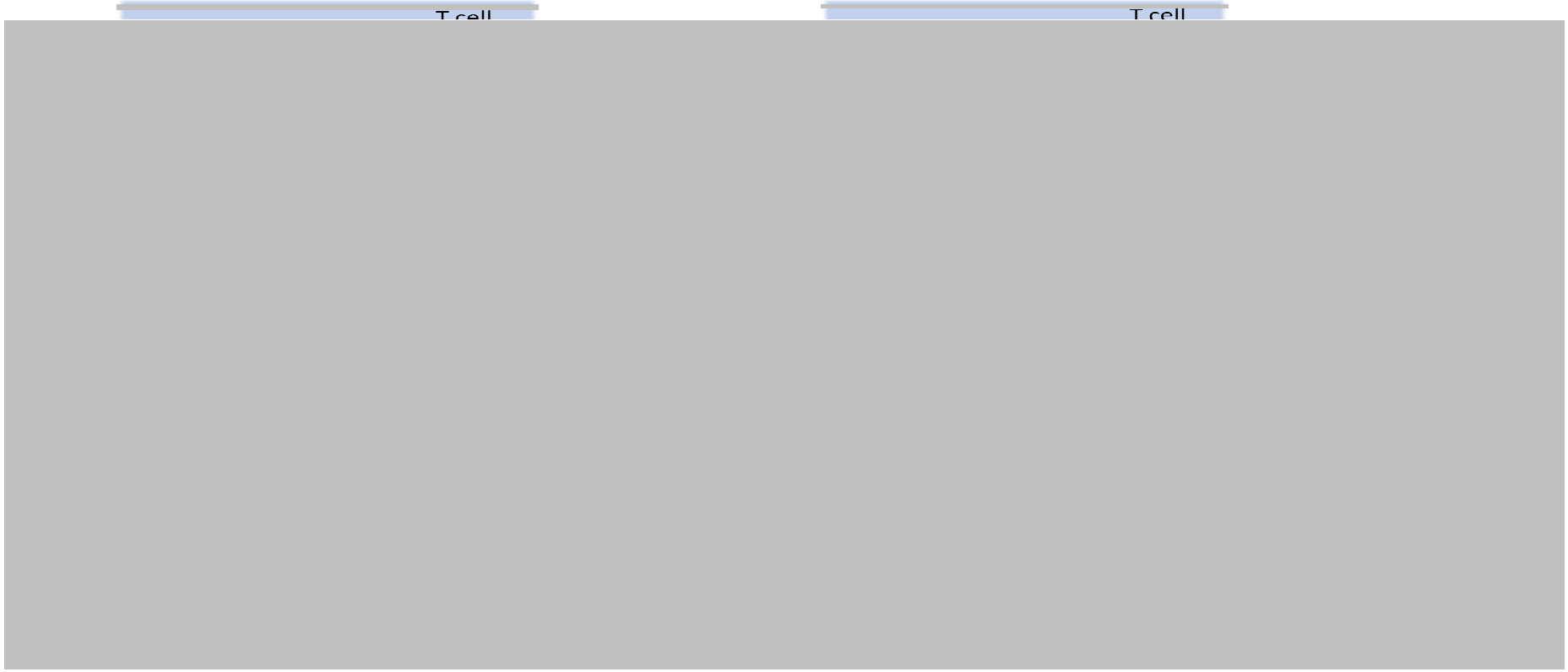
1.4.2 T-cell activation by superantigens

Conventional antigens are processed within antigen presenting cells into peptide fragments, which are then loaded into human leukocyte antigen (HLA) class II molecules to stimulate responses in only a tiny proportion of resting T cells [7, 36]. As described above, superantigens require no intracellular processing and bind both HLA class II and the TcR in the extracellular compartment away from peptide binding regions and involving both CD4 and CD8 T cells [22, 32, 107]. This leads to massive secretion of inflammatory cytokines from the T cell and APC and Vβ specific proliferation [53]. This systemic release of cytokines such as TNFα, IL-1β, IL-2 and IL-12, in addition to profound proliferation of T cells, can result in epithelial damage, capillary leak, hypotension, multi-organ failure and acidosis and other symptoms caused by SAgs such as emesis and fever [8, 31]. SAgs may also induce upregulation of genes such as TNFα and IL-1β, causing further secretion of inflammatory cytokines [108]. Following stimulation with superantigens Vβ specific T cells may become anergic, or 'deleted' by apoptosis, leading to a skewed immune response in future exposures, and to other antigens [109, 110]. Figure 1.5 shows a comparison of conventional vs SAg antigen presentation.

Furthermore, in addition to activating large numbers of the hosts T cell repertoire, in the forming of ternary complexes between the TcR and HLA class II molecule, superantigens allow other co-stimulatory molecules on the antigen presenting cell to come into close contact with their respective ligands on the T cell membrane surface. This results in further signal transduction into the T cell, triggering further upregulation of expression of pro-inflammatory cytokine genes, as well as upregulation of the expression of co-stimulatory molecules [107].

Figure 1.6 Conventional vs Superantigen Presentation

Adapted from Figure 1 Llewelyn *et al* 2002 [8]



Adapted from Figure 1, Llewelyn et al The Lancet Infectious Diseases Vol 2 March 2002 p156-162

1.4.3 Other superantigen properties

Bacterial superantigens have several other effects than those already described. These will be briefly discussed here.

1.4.3a Emesis

Many staphylococcal SAgs can trigger emesis at concentrations as low as 1µg [77]. The precise mechanism behind how they elicit this effect is unknown, although a loop structure found in many of the emetic staphylococcal superantigens has been proposed to have a role in this property. This loop is formed by a disulphide bridge between 2 cysteine residues between the two terminal domains, and may vary in length from 9 residues (SEA) to 19 (SEA) [33, 89]. It has been hypothesised that the emetic effects of these toxins may be due to interactions with cells other than T cells. For example, SE interaction with mast cells may stimulate the release of leukotrienes or prostaglandins, which may contribute to emesis [111, 89, 112-114].

1.4.3b Augmentation of endotoxin activity

It has long been recognised that superantigen and LPS can act synergistically – i.e. the superantigen can augment the activity of the bacterial endotoxin, lipopolysaccharide (LPS) [115]. Studies conducted when sub-lethal doses of superantigen and LPS were administered to rodents simultaneously have shown that shock can be induced. Lethal doses of LPS and SAg are reduced by between 100 fold to 50000 fold when administered together [116, 117]. The two proteins also appear to induce lethal TNFα shock when administered in together in such low doses [117] The mechanism behind this is not entirely understood at present, although it has been hypothesised that superantigens enhance the expression of TLR4, the endotoxin receptor, leading to increased recognition of endotoxin and thus increased production of inflammatory cytokines associated with sepsis [31, 116].

1.4.3c Local Inflammation

Aside from their potent superantigenic effect on T cells SAgs have also been shown to recruit neutrophils in mice, increasing local inflammation [118]. The mechanism behind this is thought to be secretion of TNFα from monocytes, which causes the upregulation of adhesion molecules such as ICAM. This in turn aids neutrophil migration and adhesion, recruiting neutrophils to the site of infection [118]. How important this is in the aetiology of human disease remains to be determined. However in mice, which show generally less pronounced responses to SAgs, infection with strains lacking *spea* results in a poorer outcome than infection with strains carrying the gene. This is likely to be a result of a more pronounced neutrophil response in the absence of SPEA [118, 119].

1.4.4 Streptococcal M protein

M protein is a cell wall component of *S. pyogenes* formed of a super-coiled coil α-helix structure [120]. Many different isotypes of M protein exist in group A and C streptococcus. A role for certain M protein types in rheumatic fever exists (e.g. M18, M5, and M89) [120]. M protein bears a striking structural similarity to cardiac myosin and infection with a rheumatogenic strain of *S. pyogenes* carrying SAg genes could lead to proliferation of T cells including autoreactive subsets. These T cells may then respond to the self antigen cardiac myosin due to molecular mimicry, leading to myocarditis and rheumatic fever [120-122]. However the role of M protein as a 'superantigen' remains a contentious point. There is some limited evidence that purified M protein can induce Vβ specific proliferation of T cells [123], however other evidence exists which indicates this Vβ specific proliferation is due to contamination with pyrogenic exotoxin [124].

1.4.5 B cell superantigens

In addition to the classical T cell superantigens described, additional bacterial proteins have been named B cell superantigens. These proteins cause B cell proliferation and release of inflammatory cytokines [125] and bind the B-cell receptor, immunoglobulin at sites distant to the antigen binding region. Proteins thus classified as B cell superantigens include Protein A from *S. aureus* and Protein G from *S. pyogenes,* both of which have long been known to bind immunoglobulin with high affinity, and which have been used commercially for high purity immunoglobulin purification for many years [125-127]. A third B cell superantigen is Protein L from *Peptostreptococcus magnus* which binds κ light chains of immunoglobulin and elicits similar effects on B cells to Protein A and Protein G [128, 129]. Short peptides of Protein L have been developed for the capture of immunoglobulin molecules [129].

1.4.6 Mouse Mammary Tumour Virus and Endogenous Superantigens

1.4.6a Mouse Mammary Tumour Virus

Although this research focuses on the bacterial superantigens and in particular those of *S. pyogenes* and *S. aureus,* it is important to note that superantigens have also been isolated from other sources. Although superantigens were not termed as such until the late 1980s, superantigenic properties were discovered in minor lymphocyte stimulating (Mls) antigens as early as 1973 [8, 130-132]. Mls, which are found in murine thymic stromal cells, are endogenous to the genome and stimulate Vβ specific expansion of T cells in a similar manner to bacterial superantigens. The expanded T cell population is subsequently deleted, resulting in holes in the T cell Vβ repertoire, which is observed in mice born carrying these genes. Further investigation into these endogenous superantigens revealed that the genes arise from integration into the murine genome from the mouse mammary tumour virus (MMTV) genome [130, 133]. The MMTV superantigen has a critical role in the MMTV life cycle. MMTV infects murine B

cells and produce the MMTV superantigen. In mice which do not carry endogenous superantigen genes, this causes a vast expansion in Vβ specific T cells to 'help' the infected B cells by promoting B cell proliferation [7, 134]. However this actually results in a massive increase in viral load and an infection is established [130, 132, 135]. Mice carrying the endogenous superantigen genes do not respond in this way to MMTV infection as they lack the Vβ T cells responsive to the MMTV superantigen due to previous expression of the Mls and subsequent deletion of the responding Vβ T cells [8, 130-134].

1.4.6b Viral superantigens affecting man

Extensive research has revealed that the human Vβ repertoire does not contain holes, however although no human equivalent of Mls have been identified to date, it has become apparent in recent years that human endogenous superantigens do exist. The human endogenous superantigens arise from an integration of the *env* gene into the human genome from the endogenous retrovirus HERV K18 [136] [8]. Under normal circumstances this gene is not expressed at any significant level, but infection of cells with Epstein Barr Virus (EBV) causes this gene to be upregulated. It is possible that EBV uses this superantigen to expand a population of T cells where latency can be established, a theory which could possibly also apply to the Vβ specific expansion of T cells observed in HIV, rabies, or cytomegalovirus infection [8, 136].

1.5 Superantigens in human disease

Whilst SAgs are wide-spread and most *S. aureus* and *S. pyogenes* strains are toxigenic, most adults have neutralising antibodies present in their sera for a number of SAgs, suggesting contact with these agents is more common than one may expect [7, 8]. Despite this the toxins have been implicated in the aetiology of a wide spectrum of disease, ranging from pharyngitis to toxic shock syndrome. Differences in magnitude of response to SAgs have been attributed at least in part to HLA class II polymorphisms [65-67, 137]. Toxic shock syndrome and other diseases with hypothesised SAg roles are discussed here.

1.5.1 Toxic shock syndrome

Toxic shock syndrome (TSS) is a capillary leak syndrome which was first described in 1978 in seven children presenting with high fever, hypotension, multisystem involvement and an erythematous rash. Desquamation of the skin followed [138]. These features now characterise the syndrome. Shock in TSS is TNFα induced [139]. This new syndrome was attributed to *S. aureus* colonisation and infection and subsequently a new toxin was identified from this strain of *S. aureus.* This toxin would later be named toxic shock syndrome toxin 1 (TSST-1) [138]. In the 1980s TSS was particularly associated with menstruating women and aetiology of disease attributed to the use of tampons. Changes in the manufacture of tampons and education of women using them saw a reduction in menstrual TSS. However cases of non-menstrual TSS continued to emerge often associated with staphylococcal infection following severe burns or surgery [140]. In the late 1980s a similar syndrome was described by Cone in patients with streptococcal infections such as cellulitis, necrotising fasciitis or pharyngitis. This was termed streptococcal toxic shock syndrome (sTSS) [141]. The clinical features of staphylococcal and streptococcal toxic shock syndromes differ slightly and are shown in Table 1.5.

Originally it was believed that TSS cases were seen in patients colonised or infected with particularly virulent strains of *S. aureus* and *S. pyogenes* however due to the high level of horizontal gene transfer between bacteria, and the mobile genetic location of these toxin genes, most strains of *S. aureus* and *S. pyogenes* have been shown to be toxigenic [8, 11, 12]. Therefore the spectrum of disease seen with these toxins has since been attributed at least partially to HLA class II polymorphisms [65-67, 137]. Additionally, there is evidence to suggest that different superantigens are produced under different conditions. For example enterotoxin gene cluster (*egc*) toxin genes in *S. aureus* are more prevalent in commensal strains than invasive strains [142] and when present appear to be inversely correlated with the severity of sepsis [142, 143]. The opposite is true of non-*egc* staphylococcal superantigens. Furthermore *egc* superantigens are secreted during exponential growth, whilst non-*egc* superantigens are secreted in the late stationary phase [143]. Although

superantigens are classically presented by 'professional' antigen presenting cells such as dendritic cells and monocytes, evidence exists for the presentation of superantigens by other cell types such as natural killer cells (NK cells) which have been shown to activate between 4% and 16% of T cells in response to SEB [144]. Additional cell subsets such as smooth muscle airway cells have been shown to present SAgs with the effect of exacerbating proasthmatic conditions in rabbit tissue [145]. Expression of different SAgs under different conditions, as well as presentation of SAgs by non-professional APCs also contribute to the spectrum of disease observed with superantigen exposure.

Since the original description of TSS and sTSS superantigens have been shown to be the causative agents for menstrual and non-menstrual toxic shock syndrome, both staphylococcal and streptococcal (TSST-1, SPEA, SEA,) [8]. The involvement of staphylococcal superantigens in toxic shock syndrome was originally shown by De Azavedo and co-workers who devised a rabbit uterine model in which various strains of *S. aureus* were implanted into rabbits to induce menstrual toxic shock. Those rabbits infected with strains expressing TSST-1 developed toxic shock syndrome symptoms, whilst those infected with TSST-1$^-$ strains did not [146]. Similar models have since been used to show the involvement of other staphylococcal superantigens in TSS (SEA, SEC1, SEB) [147-149] and streptococcal superantigens SPEA and SPEC [150]. Additionally, Vβ specific expansions of T cells bearing Vβs 2 and 8 have been observed in TSS mice [151, 152]. As described above, Vβ specific proliferation of T cells is a marked characteristic of superantigen mediated disease.

The mechanism behind the progression of TSS and sTSS is believed to be a cytokine storm, induced by a massive over-reaction of the host's immune response. As superantigens bind HLA class II and the T cell receptor away from the specific peptide binding regions they can stimulate proliferation of up to 50% of the hosts T cell repertoire, resulting in a massive systemic release of inflammatory cytokines. This can lead to capillary leak, hypotension and consequentially other clinical features seen in TSS [10].

TSST-1 remains the superantigen most commonly associated with menstrual TSS which is usually attributed to the unique ability of TSST-1 to cross mucosal barriers [149], although SEB has recently also been shown to have this ability to a lesser extent [153]. Non-menstrual TSS is more usually associated with staphylococcal toxins such as SEA and SEC which cannot cross mucosal barriers [149], or streptococcal superantigens SPEA or SPEC [31, 149, 150]. Additionally SPEA and SPEC are implicated in the aetiology of scarlet fever [31].

Table 1.6 Clinical features of staphylococcal and streptococcal TSS

Data broadly derived from Llewelyn *et al* 2002, Stevens 1996 and Lappin 2009 [8, 140, 154]

	Staphylococcal TSS	**Streptococcal TSS**
Clinical presentation	fatigue fever >38.9°C chills diarrhoea confusion myalgia tachycardia, hypotension, erythema and oedema adult respiratory distress syndrome desquamation at 7-14 days superficial infection site – burns, nappy rash, surgery etc usually focal infection	fatigue fever diarrhoea confusion myalgia tachycardia, hypotension, oedema and erythema adult respiratory distress syndrome severe pain desquamation - rare deep sited infection – necrotising fasciitis, myositis, septic joint sometimes multiple infection sites
Pathology	neutrophilia, thrombocytopenia, renal impairment hypoalbuminaemia deranged coagulation transaminitis bacteraemia (rare)	neutrophilia thrombocytopenia, renal impairment hypoalbuminaemia deranged coagulation transaminitis bacteraemia (common)
Mortality	~6%	30-70%

1.5.2 Food poisoning

The staphylococcal SAgs are responsible for staphylococcal food poisoning. Whilst the novel loop structure described above is believed to be partly responsible for emesis in food poisoning [89], it is also believed that staphylococcal SAgs may stimulate the vagus nerve, triggering further emesis through the release of serotonin [155]. An important characteristic of these toxins is their ability to withstand denaturing conditions, both heat, with temperatures of up to 120°C necessary for denaturation, and pH. This means the SAgs are able to withstand both normal cooking processes and denaturing conditions once ingested, which may contribute to their ability to cause food poisoning [156].

1.5.3 Kawasaki's Disease

Kawasaki's Disease (KD) is an acute vasculitis in children. It presents with high fever, erythematous rash, conjunctivitis, inflamed mucous membranes and lymphadenopathy [157], clinical features which at original presentation may be mistaken for TSS [9]. That the disease is primarily of children suggests involvement of a widespread bacterial agent which adults may have acquired a level of immunity to [9]. The disease is associated particularly with an expansion of Vβ2 positive T cells, indicative of a superantigen mediated process [158, 159]. In addition to Vβ responses observed in KD toxin producing strains of *S. aureus* and *S. pyogenes* have been isolated from KD patients. Strains of *S. aureus* producing TSST-1 and *S. pyogenes* producing SPEC were isolated, although these findings have not been consistent, suggesting that SAgs may not be required for disease progression. One hypothesis that has been suggested for the role of SAgs in KD is that patients are colonised with SAg producing strains of *S. aureus* or *S. pyogenes* in mucosal surfaces and secreted SAgs are absorbed through the inflamed surface. This may then cause an expansion of Vβ2 T cells, including possible autoreactive T cells. The autoreactive T cells may then enter the vascular system and heart tissue causing release of cytokines and inflammation. This may then develop into the myocarditis and vasculitis seen in KD [9, 160]. However, the role for SAgs in KD is by no means fully confirmed and remains to be elucidated further.

1.5.4 Rheumatic Fever

Rheumatic fever (ARF) is a disease which usually follows pharyngeal infection with *S. pyogenes* in children and which is the leading cause of preventable paediatric heart disease [97]. Certain M types of *S. pyogenes* have been termed rheumatogenic (M1, 3, 5, 18 and 89) [97, 120, 161] and are associated with acute rheumatic fever. While the molecular mimicry mechanism of streptococcal M protein appears to play a clear role in the aetiology of ARF [120, 121], it has also been hypothesised that streptococcal superantigens may contribute towards the disease progression [7, 58, 97, 161]. Recently strong evidence has been provided of this. SPEL and SPEM were identified in rheumatogenic M3, M18 and M89 strains of *S. pyogenes* [7, 58, 97, 161-163]. These toxins induce Vβ specific proliferation of

T cells *in vitro* [7, 161]. Importantly, anti SPEL and SPEM antibodies were also identified in the sera of convalescent ARF patients but not in sera from patients infected with non-rheumatogenic strains, indicating the role these toxins may play in the disease [161, 164].

1.5.5 Rheumatoid arthritis, psoriasis, atopic eczema and dermatitis

In addition to the more established roles of SAgs in toxic shock syndrome, as well as food poisoning, KD and ARF, SAgs may also contribute to diseases such as acute guttate psoriasis, atopic dermatitis (AD) and rheumatoid arthritis (RA).

Acute guttate psoriasis is a chronic inflammatory skin disease affecting around 2-3% of the population [9, 165]. Infiltration of inflammatory cells such as neutrophils and proliferation of keratinocytes leads to the distinctive erythematous plaques seen in this disease [9, 165]. Skin biopsies from these plaques have been demonstrated to be Vβ2 skewed in both the CD4 and CD8 T cells. Additionally streptococcal isolates from psoriasis patients have been demonstrated to express SPEC [165]. Further, application of TSST-1, SEB, SPEA and SPEC to clinically uninvolved abraded skin of psoriasis patients has been shown to induce formation of plaques, however in this case Vβ skewing was not seen. Instead, keratinocytes in psoriatic patients appeared to express far higher levels of HLA-DR than healthy controls, which is hypothesised to enhance SAg induced inflammation [166].

S. aureus can be isolated from 90% of chronic skin lesions from patients with atopic dermatitis. Application of superantigen to unaffected atopic skin has been demonstrated to cause a 'flare up', which is further increased if superantigen is applied to broken skin. It has been found that keratinocytes, which usually do not express HLA class II, are induced to express HLA class II during inflammation which may allow superantigens to elicit their effects [167]. Primarily Th2 cells seem to be activated by this mechanism, which are thought to be important in the initiation of atopic lesions [167]. Additionally, in patients with AD elevated levels of IgE for TSST-1, SEB or SEA are found. Basophils from patients with this IgE degranulate when treated with toxin. This led to the hypothesis that mast cells and basophils in AD patients degranulate in the presence of SAg, releasing histamines and initiating the scratch cycle of AD [9, 167].

Rheumatoid arthritis is an autoimmune disorder of joints characterised by chronic and recurring inflammation in multiple joints [168]. The precise mechanisms behind this complex disease are not entirely understood, although certain HLA-DR alleles have been associated with the disease, namely HLA-DR4 homozygous. Evidence for superantigen involvement in this disease is limited [168]. Synovial monocytes of RA patients were found to be significantly more Vβ14 skewed than in peripheral blood. Also, as is the case with many autoimmune disorders connected to superantigens, the expansion of large subsets of T

cells increases the chance of autoreactive T cells being expanded, which increases the possibility of autoimmunity [168].

1.5.6 Superantigens in autoimmunity

Two main mechanisms exist for the hypothesised role of superantigens in autoimmune disorders. Firstly, as superantigens cause such massive proliferation of T cells, the likelihood that self reactive T cells will be among those which proliferate is quite high [169]. This would potentially allow these T cells to come into contact with their respective self antigens, which leads to the possibility of inflammation [170].

The second proposed mechanism for the involvement of SAgs in autoimmunity is that of molecular mimicry. Whilst it is unknown if the classical superantigens share a structure similar to host proteins, and therefore if they could elicit autoreactive T cell responses, other superantigens have been shown to have structural homology with host antigens. MAM for example bears a structural similarity to collagen, and is associated with exacerbation of collagen induced arthritis in mice [170]. Additionally, although the classification of streptococcal M protein as a superantigen remains contentious, the protein does bear striking structural resemblance to cardiac myosin. Antibodies to this protein cross-react with self cardiac myosin, and therefore may contribute to the inflammation and immune responses seen in the autoimmune disease ARF [170].

1.6 Aims of Project

The range of SAgs associated with *S. aureus* and *S. pyogenes* indicates their biological importance to these major human pathogens. Nevertheless a huge amount remains unknown about these toxins including their real biological purpose and their role in human diseases such as sepsis (more generally than TSS), autoimmunity and atopy. The study of superantigens is significantly limited by availability of reagents for their detection both in the laboratory and clinically. As has been described they are highly serologically diverse and new toxins continue to be discovered in streptococcal and staphylococcal genomes. Also they are biologically active at picomolar to fentomolar concentrations. While Vβ specific changes in T cell repertoire are often considered diagnostic of superantigenicity, such changes are inconsistent even in TSS. This is because Vβ responses are not only species specific (thus problematic in animal models) but also HLA class II background, concentration and probably time course of exposure play a role in the aetiology of disease.

The fact that superantigen interactions with HLA class II are so highly restricted to two well characterised and relatively conserved sites coupled with the high affinity of these interactions (up to 10^{-7}M) raises the possibility that HLA class II – or structures based on the superantigen binding domains of HLA class II – could be exploited to develop novel diagnostic tools for the study of bacterial superantigens.

This research aimed therefore firstly to obtain proof of concept the HLA class II binding of SAgs is of sufficient affinity and specificity that HLA class II could be used in this way.

This was divided into the following individual aims:

- Generate HLA class II or HLA class II based ligands using either affinity purification, expression systems or a proteomics approach
- Express a range of recombinant SAgs
- Early binding assays utilising the two molecules.

Throughout this research, particularly in the case of expression of streptococcal recombinant SAgs additional observations were made regarding differing superantigenic responses between individuals. This led to the secondary aims:

- Characterise the Vβ signature of novel and classical recombinant streptococcal SAgs
- Investigate the influence of HLA-DR polymorphisms on the Vβ responses to SPEM.

2. Materials and Methods

2.1 General Materials

General materials used in this study are detailed below. Any buffers specific to one method, or more complex buffers, are described in subsequent chapters. All buffers used in columns were degassed for 30 minutes and filtered through 0.45µM filters before use.

2.1.1 Standard media, buffers and solutions

All chemicals were from Sigma-Aldrich, St Louis, MO unless otherwise stated.

Table 2.1 Buffers and solutions

4% polyacrylamide stacking gel	One gel: 3.05ml dH_2O, 1.25ml 0.5M TrisHCl pH 6.8, 50µl 10% SDS, 650µl 30% Bis-Acrylamide (Severn Industrial, UK), 50µl 10% APS, 10µl TEMED added last to polymerise gel
12% polyacrylamide resolving gel	One gel: 3.5ml dH_2O, 2.5ml 1.5M TrisHCl pH 8.8, 100µl 10% SDS, 4ml 30% Bis-acrylamide, 50µl 10% APS, 10µl TEMED
SDS-PAGE Running Buffer	25mM Tris, 192mM glycine, 0.1% SDS
SDS-Stock Buffer	4% SDS, 0.1M Tris, 2mM EDTA, 0.1% bromophenol blue, 20% glycerol, 0.25M DTT (added fresh before use) pH 8.9
Coomassie Blue Stain	0.25% Coomassie Brilliant Blue (Sigma-Aldrich) in 10% glacial acetic acid, 40% absolute ethanol
Destain Solution	40% methanol, 7% glacial acetic acid
BSA Protein Standard (Thermo Fisher Scientific)	1mg/ml BSA diluted with dH_2O in 200µg steps

Complete RPMI	1 x RPMI (GIBCO, Carlsbad, CA) supplemented with 10% FCS, 2mM L-glutamine, 20U/ml penicillin and 20µg/ml streptomycin.
Complete DMEM	1 x DMEM (GIBCO) supplemented with 10% FCS, 2mM L-glutamine, 20U/ml penicillin and 20µg/ml streptomycin
1 x Tris-acetate buffer (TAE)	40mM Tris acetate, 1mM EDTA pH8.0
Agarose gel	1-2% agarose, 0.01% GelRed (Cambridge BioSciences
10 X Phosphate Buffered Solution (PBS):	1.7M NaCl, 0.3M KCl, 0.1M Na_2HPO_4, 20mM KH_2PO_4
1 X PBS	10% 10 x PBS
Dialysis PBS	1 x PBS 0.05% sodium azide
ELISA Wash buffer	1 X PBS 0.05% Tween-20
ELISA zinc wash buffer	1 X PBS 0.05% Tween-20, 8µM $ZnCl_2$
ELISA EDTA wash buffer	1 X PBS 0.05% Tween-20, 2mM EDTA
ELISA Blocking Solution	1 X PBS/3% BSA
ELISA zinc blocking solution	1 X PBS/3% BSA, 8µM $ZnCl_2$
ELISA EDTA blocking solution	1 X PBS/3% BSA, 2mM EDTA
ELISA Stopping solution	2N H_2SO_4
Luria Bertani Broth (LB) (Thermo Fisher Scientific)	1% bacto-tryptone, 0.5% bacto-yeast, 1% NaCl 200µg/ml pH 7.0, 100µg/ml ampicillin

Luria Bertani Agar	LB 1.5% agar, 100µg/ml ampicillin
IPTG	100mM stock. Used at 1mM for gene induction
FACS Buffer	1 x PBS, 1% BSA, 0.05% NaN_3
FACS zinc buffer	1 x PBS, 1% BSA, 8µM $ZnCl_2$ 0.05% NaN_3
Tryptone Soya Broth (TSB)	30g TSB powder dissolved in 1L dH_2O and autoclaved before use
Lysostaphin (Sigma-Aldrich)	1mg powder in 200µl 20mM sodium acetate
Sodium acetate	20mM sodium acetate
PreScission Protease buffer	50mM Tris-HCl pH 8.0, 150mM NaCl, 10mM EDTA, 1mM DTT, 20% glycerol.
SOB Medium	2% tryptone, 0.5% yeast extract 8.5mM NaCl, 10mM $MgCl_2$ 10mM $MgSO_4$.

2.1.2 Antibodies

Antibodies were used in ELISAs, flow cytometry and in columns to purify HLA-DR. Tables 2.2 and 2.3 list the antibodies used.

Table 2.2 Antibodies

Name of hybridoma/source	**Specificity**	**Manufacturer**
HB95 hybridoma cell line (W6/32)	HLA class I	American Tissue Culture Collection (ATCC)
HB55 hybridoma cell line (L243)	HLA-DR α chain	ATCC
Rabbit ascites fluid	Streptococcal pyrogenic exotoxins C (SPEC)	Toxin Technology Inc. (Sarasota, FL)

Table 2.3 Antibody conjugates and secondary reagents

Name of hybridoma/ source	**Specificity**	**Conjugate**	**Manufacturer**
Goat ascites fluid	Rabbit IgG (H+L)	HRP (LightningLink HRP Kit, Innova Biosciences)	Immunopure Pierce (Perbio, UK)
TDR31.1	Pan-MHC class II β chain	Biotin	Ancell Alexis Corporation
HB95 hybridoma cell line (L243)	HLA-DR α chain	HRP (as above)	ATCC Manassas VA
Streptomyces avidinii (Streptavidin)	d-biotin	HRP	AbD Serotec (UK)
BL37.2	Human TCR Vβ1	Phycoerythrin (PE)	Beckman Coulter
MPB205	Human TCR Vβ2	PE	Beckman Coulter
IMMU157	Human TCR Vβ5.1	PE	Beckman Coulter
56C5.2	Human TCR Vβ8	PE	Beckman Coulter
VER2.32.1	Human TCR Vβ12	PE	Beckman Coulter
CAS1.1.3	Human TCR Vβ14	PE	Beckman Coulter
IMMU546	Human TCR Vβ22	PE	Beckman Coulter
AF23	Human TCR Vβ23	PE	Beckman Coulter
RPA-T4	Human CD4	FITC	BD Pharmingen
TMB easy liquid substrate	Substrate for HRP	n/a	Thermo Fisher Scientific

2.1.3 Cell Lines

2.1.3a Human cell lines

Human EBV transformed B cell lines homozygous for specific HLA-DR types were grown from frozen stocks donated by Professor Daniel Altmann, Department of Infectious Diseases and Immunology, Imperial College London. Cells were cultured in 75cm^2 flasks and maintained in complete RPMI. Cells were passaged every 2-3 days and harvested at 3-5 x 10^6 cells/ml by centrifugation after ~14 days. Cell pellets were frozen at -80°C. Table 2.4 summarises the cell lines used.

Table 2.4 Human EBV transfected B-cell lines

Cell line	***DRB1****	***DQA1****	**Serotype**
PGF	*15011*	*01021*	DR15 DQ6
WT51	*04011*	*0301*	DR4 DQ8

2.1.3b Hybridoma cell lines

Monoclonal antibodies were purified from the supernatant of hybridoma cell lines.

- HB95 murine hybridoma cell line (ATCC). Cells were maintained in complete DMEM for one week, adjusted to Hybridoma-Serum free medium (Hyb-SFM, Invitrogen Carlsbad CA) over one week and maintained in this medium until harvesting volumes of 2-3L.
- HB55 murine hybridoma cell line (ATCC, Manassas VA). Cells were maintained in complete RPMI over one week, adjusted to Hyb-SFM over three days and maintained in this medium until harvesting at volumes of 2-3L.

Table 2.5 Hybridoma cell lines

Hybridoma cell line	**Antibody produced**	**Specificity of antibody**
HB95	W6/32	HLA class I
HB55	L243	HLA-DR α chain

2.1.3c Bacterial cell lines

Bacterial cell lines were used for extraction of bacterial genomic DNA and transformations with complete expression vectors. Table 2.6 summarises the bacterial cell lines used.

Table 2.6 Bacterial cell lines

	Application	**Manufacturer**
ATCC 13565 (*Staphylococcus aureus*)	Extraction of bacterial genomic DNA for amplification of *sej*	ATCC VA
DH5α (*Escherichia coli*)	Transformation with pPRO-EX HTb encoding SEJ	Stratagene CA
XL1-Blue (*E. coli*)	Transformation with pPRO-EX HTb encoding SEJ	Stratagene CA
TOP10 chemically competent (*E. coli*)	Transformation with Topo pCR 2.1 encoding SEJ	Invitrogen CA
BL21 pLys (DE3) (*E. coli*)	Transformation with pGEX-3c and pET32a encoding SPEC, L and M	Stratagene CA
BL21 (*E. coli*)	Expression of recombinant SPEA in pET19 vector	Transformed *E. coli* donated by Professor Shiranee Sriskandan

2.1.4 Toxins

Toxins used in this study were either purchased (SPEA, SEA and SPEC) or expressed in *E. coli* using plasmid expression vectors. Recombinant SPEA was expressed using transformed *E. coli* BL21 which were donated by Professor Shiranee Sriskandan (Department of Infectious Diseases, Imperial College, London). Recombinant SPEC, SPEL and SPEM minus signal peptides were expressed as fusion proteins from plasmids donated by Professor Thomas Proft (Department of Molecular Medicine and Pathology, University of Auckland). Professor Proft also donated a plasmid encoding full length picornavirus protease 3C for removal of protein tags. This protease cleaves at EVLFQ|GP [97]. Table 2.7 details the toxins and vectors used.

Table 2.7 Toxins and expression systems

Protein	Size (kDa)	Vector	Fusion Partner/tag	Size plus tag (kDa)	Source
SEA	27.1	n/a	n/a	-	Toxin Technology Inc, Sarasota FL
SPEA	26.0	n/a	n/a	-	ToxTech
SPEC	24.4	n/a	n/a	-	ToxTech
rSPEA	26.0	pET19b	His-tag	26.0	Professor S Sriskandan
rSPEC	24.4	pGEX-2T	GST	46.4	Prof T Proft
rSPEL	27.4	pET32a-3c	Thioredoxin (Trx) containing an internal patch of histidines	49.4	Prof T Proft
rSPEM	26.2	pET32a-3c	Trx	48.2	Prof T Proft
3C	22.0	pGEX-3c	GST	44.0	Prof T Proft

2.2 General Methods

General methods used in more than one chapter are described below. All methods specific to one set of results are described in the following chapters.

2.2.1 Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate proteins based on their molecular weight to determine the presence of HLA-DR in column eluents and recombinant SAgs in bacterial lysates.

12% resolving gels were cast in Mini-PROTEAN® Cell Assembly apparatus' (Bio-Rad Laboratories) and overlaid with saturated iso-butanol to ensure a smooth interface. These set for 30 minutes at room temperature (RT). Saturated iso-butanol was rinsed from the gels and 4% stacking gels cast on top. Wells were created using plastic combs and gels set at RT. Plastic combs were removed and the gels assembled into Mini- PROTEAN® electrophoresis running tanks. Inner reservoirs were filled and outer reservoirs half filled with SDS-PAGE running buffer.

10µl samples and negative controls were prepared by addition of SDS stock loading buffer and boiling for 5 minutes. Low molecular weight markers, ColorBurst Electrophoresis Markers (Sigma-Aldrich St. Louis, MO) and samples, were loaded and gels run at 200V for 1 hour.

Gel Staining

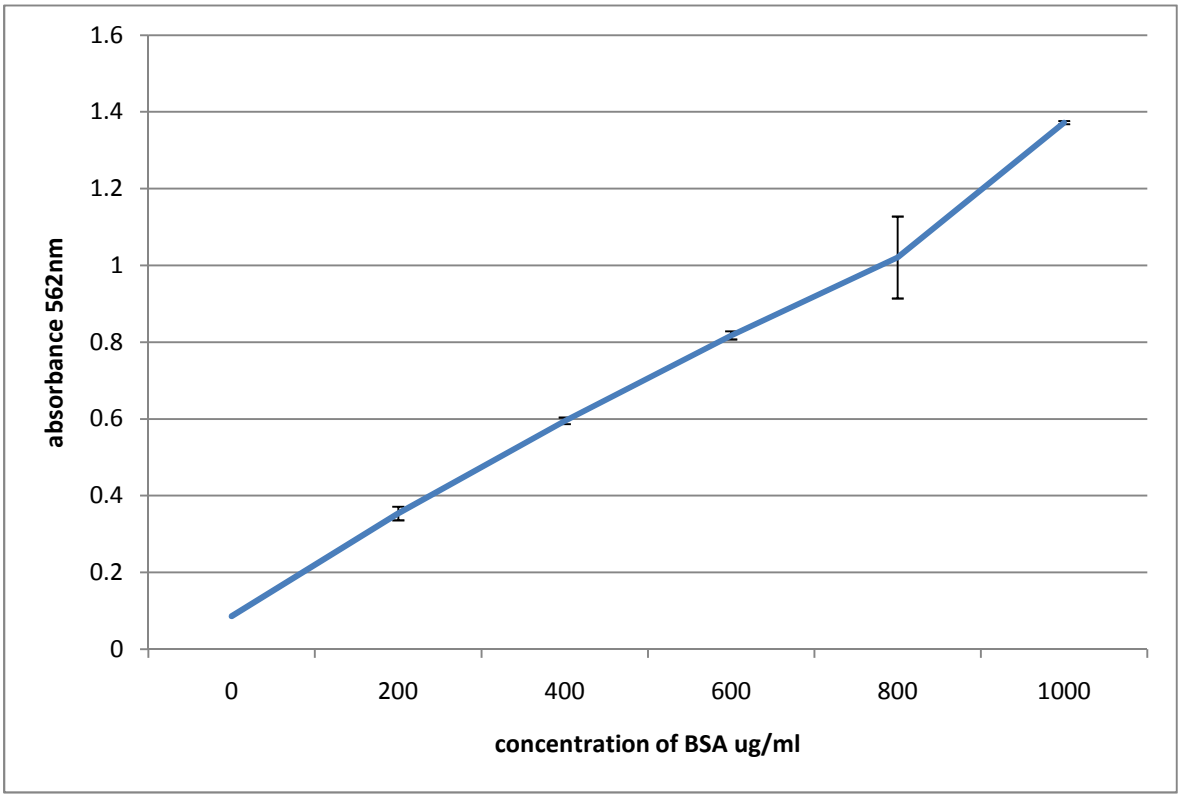
Gels were visualised by submersion in coomassie blue overnight at 4°C. Gels were destained by submersion in destain solution with rocking. Destain solution was changed at intervals and destaining continued until protein bands were visible. Gels were dried in cellophane, and bands compared with low molecular weight markers for size.

2.2.2 BCA Protein Estimation

Protein estimations were conducted in 96 well microtitre plates using the Bicinchoninic Acid Protein Assay kit (Sigma-Aldrich, St Louis, MO) according to manufacturer's instructions. Bovine serum albumin (BSA) standards were prepared and 25µl plated in duplicate alongside samples and blanks. 200µl working reagent prepared as a 1:50 ratio of Reagent B: Reagent A was added to each well and purple colour developed for 30 minutes at 37°C. Absorbance was read in a Synergy plate reader using KC4 software at 562nm and the results plotted. Sample concentrations were calculated using the standard curve. Purity of samples was analysed using SDS-PAGE. This protein assay kit can accurately assess total protein concentration between 200-1000µg/ml protein in samples measured against known standards as shown in Figure 2.8 below.

Figure 2.1 A BSA standard curve.

Points shown are the mean of duplicate readings +/- 1 standard deviation. Protein concentrations were calculated using this or a similar standard curve.



2.2.3 Isolation of PBMCs

Peripheral blood monocyte cells (PBMCs) were used in superantigen Vβ proliferation assays. Healthy donor blood was drawn with informed consent using the Vacutainer© system (ethics in place Life Sciences, University of Sussex). Whole blood was diluted in an equal volume of complete medium and overlaid on top of 20ml Ficoll-Paque (GE Healthcare, UK).

Blood gradients were centrifuged (1800 rpm, 35 minutes) with low acceleration/deceleration. Buffy coat was harvested and washed three times in complete medium. Cells were counted using a Haemocytometer.

2.2.4 Flow Cytometric Vβ profile analysis of superantigen stimulated PBMCs

Flow cytometric analysis was used to validate the biological properties of recombinant superantigens. An *in vitro* proliferation of donor PBMCs and analysis of Vβ response over a range of concentrations was conducted in each case as follows

1 x 10^6/well PBMCs were seeded in a 24 well plate. Cells were stimulated with either PHA as a positive control or SAgs a range of concentrations. Stimulated cells were incubated (37°C, 5% CO_2, 72 hours) before washing three times in FACS buffer. Cells were double-stained on ice with 2µl anti-CD4 FITC antibody and 2µl anti-Vβ PE antibodies (Table 2.3) for 1 hour. Samples were washed three times in FACS buffer and analysed by FACS.

FACS analysis was carried out using an LSR flow cytometer (Becton Dickinson USA) and FACSDiva software. Populations were gated on using unstained cells whilst compensations between yellow-green FITC fluorescence and red PE fluorescence were calculated using single stained compensation beads (BD, USA).

2.2.5 Statistics

Unpaired t-tests using two tailed analysis were used for comparison of parametric data assuming normal distribution. P-values of <0.05 were considered significant. Analysis was carried out using GraphPad Prism 5 software, GraphPad Software Inc, La Jolla CA.

3. Expression and validation of recombinant superantigens

3.1 Introduction

This work aimed to generate the reagents needed to develop binding assays and characterise TcR Vβ signatures. There is a very restricted range of commercially available SAgs and they are expensive, therefore expression of recombinant proteins is by far the best method of generating large quantities and a wider variety of these toxins. Streptococcal superantigens SPEA, C, L and M were expressed using methods described below. Attempts were also made to clone a novel staphylococcal superantigen, SEJ.

SPEA and SPEC are classical superantigens, having well defined Vβ profiles and potencies. SPEA causes an expansion in Vβs 2.1, 3.1, 13.1 and 14.1 [73], whilst SPEC reportedly targets Vβs 2.1, 3.2, 12.5 and 15.1 [58, 69]. Both have been crystallised [23, 24]. SPEA is reported to preferentially bind HLA-DQ α chain over HLA-DR [23, 67], although zinc dependent binding has also been demonstrated with SPEA suggesting some β chain binding exists as well [23, 58]. SPEC contains two high affinity zinc dependent β chain binding sites for HLA-DR, and reportedly no α chain binding site [24]. These two superantigens therefore form a good basis for a comparison of different superantigens and their binding modes.

Some confusing nomenclature exists for SPEL and SPEM. For the purposes of this research SPEL and SPEM refer to the superantigens designated SPEK/L and SPEL/M respectively (Table 1.4b). The gene encoding SPEL, identified in an M89 strain of *Streptococcus pyogenes*, is identical to a gene isolated in an M3 strain by Beres *et al* named *speK* (now referred to as SPEK/L) [7, 162]. The same gene was also identified in an M3 strain in Japan by Ikeda *et al* and was designated *speL* [163]. SPEL/M was identified in the M89 strain, and is identical to the gene named *speL* found in an M18 strain described by Smoot *et al.* An additional gene was identified in this strain which was found in a contiguous sequence to *speL* and was named *speM* [164]. This gene is identical to one named SPEM* by Proft *et al* and will not be discussed further here [58]. Both SPEL and SPEM are strongly associated with acute rheumatic fever, [7, 56, 97, 164]. They share identical Vβ signatures, reportedly causing expansions in Vβs 1, 5.1 and 23 [7, 58, 97, 164]. The two toxins have different half maximal proliferations, with SPEL inducing half maximal proliferation at 1pg/ml, and SPEM at 10pg/ml [7]. Thus these novel superantigens form the basis for a comparison of Vβ expansions in conjunction with the two classical superantigens mentioned. Following

their expression and purification, recombinant superantigens were validated using the T cell proliferation method described in general methods above.

SEJ is a novel staphylococcal superantigen which belongs to a different subfamily of superantigens to others used in the course of this research [26, 91, 171]. The gene for *sej* has been identified in a number of *Staphylococcus aureus* and is believed to be an important virulence factor, [172, 173] however very little is known about its binding mechanisms, Vβ profile or potency, making this superantigen an ideal candidate for the generation of novel data. Attempts to clone SEJ are also described below.

3.2 Methods

Buffers used were as detailed in Table 2.1 above excepting a few specific examples detailed in individual methods below.

3.2.1 Expression and purification of recombinant superantigens SPEA, C, L and M

Tables 2.6-7 show plasmids and bacterial cells used. Dehydrated plasmids on filter paper were rehydrated in sterile TE and stored at -80°C. Fusion proteins were expressed as soluble proteins and purified under normal conditions. His-tagged SPEA was expressed as insoluble granules and purified under denaturing conditions.

Buffers

SOB Medium: 2% tryptone, 0.5% yeast extract 8.5mM NaCl, 10mM $MgCl_2$ 10mM $MgSO_4$.

2M glucose.

SOC Medium 1ml filter sterilized 2M glucose solution. SOB medium to a volume of 100ml.

His-Bind BugBuster purification kit (Novagen, WI)

GST Bind BugBuster Purification kit (Novagen WI)

3.2.1a Expression of SPEC, SPEL and SPEM

BL21 (DE3) pLysS cells were transformed with 2µl rehydrated plasmid vector alongside a pUC18 positive control according to manufacturer's instructions. 100µl cells and 1.7µl β-mercaptoethanol were mixed by swirling in 14ml round bottomed polypropylene tubes (BD Falcon) and incubated on ice for 10 minutes. 2µl plasmid expression vector or 1µl pUC18 control plasmid was added and incubated on ice for 30 minutes, heat-pulsed (42°C, 45

seconds) and incubated on ice for 2 minutes. 42°C SOC was added and reactions incubated (37°C, 1 hour). Cell suspensions were plated onto LB agar and incubated overnight. LB broth was inoculated with colonies and incubated overnight at 30°C with shaking.

Optical density (OD) was measured and cultures diluted to an OD of <0.5. This was incubated under the same conditions and OD measured at intervals until an OD of 0.6 was achieved. Gene expression was induced with 1mM IPTG for three hours. Bacteria were harvested by centrifugation and frozen in 0.9% saline. Induced and uninduced bacteria were analysed on SDS-PAGE for the presence of a band at the correct size (Table 2.7).

Recombinant GST tagged SPEC was purified using GSTBind resin and buffer kit. Columns were washed using 1 x GST bind/wash buffer. Frozen bacterial pellets were weighed and 5ml Bug-buster reagent added per 1g. 1µl benzonase nuclease per ml Bug-Buster was added. Pellets were resuspended and incubated at RT with shaking for 15 minutes. Insoluble inclusion bodies were removed by centrifugation at 4500 x g for 20 minutes. Supernatant was filtered and applied to GSTBind agarose columns at RT. Columns were washed with 1 x GST bind/wash buffer and eluted using 1 x GST elution buffer. GST tags were removed using 1mg/ml trypsin at 10µl/elution overnight in solution at 4°C and GST removed from solution by running the eluent over the column. GST bound to the column, whilst SPEC was collected in the eluent.

Recombinant SPEL and SPEM were purified using HisBind resin and buffer kit. Columns were washed using 7.5ml dH_2O, 12.5ml 1 x charge buffer and 7.5ml 1 x binding buffer. Bacterial extracts were prepared and applied to the column as described above. Columns were washed with 1 x binding buffer and 1 x wash buffer. Cleavage of Trx tags was achieved on the column using PreScission protease overnight at 4°C. Eluents containing purified SPEL and SPEM were dialysed against 1 x PBS overnight. Columns were eluted using 1 x elution buffer and stripped with 1 x strip buffer before storage at 4°C. Recombinant SPEL and SPEM were analysed using SDS-PAGE and BCA protein assay and validated using a T cell proliferation assay.

3.2.1b Expression of SPEA

LB broth was inoculated with 100µl frozen stock and incubated overnight with shaking at 37°C. Bacterial extracts were prepared as above, saving the inclusion body fraction. Inclusion bodies were resuspended in 6M urea binding buffer and incubated for 45 minutes at 4°C. Following further centrifugation, supernatant contained solubilised intracellular proteins and was filter sterilised and applied to nickel charged His-Bind resin. Columns were washed with 6M urea 1 x binding buffer and 6M urea 1 x wash buffer. Bound SPEA-His-Tag was eluted in 6M urea elution buffer. Eluate was dialysed against 1 x PBS 6M urea

and urea concentration reduced over one week, allowing refolding of toxin. Following dialysis in 1 x PBS, toxin was analysed using SDS-PAGE and Bradford protein assay before validation in T cell proliferation assays and storage in aliquots at -20°C.

3.2.2 – Generation of plasmids encoding recombinant SEJ

3.2.2a Agarose Gel Electrophoresis

1.5% agarose gels were used throughout the cloning. Gels containing GelRed (Cambridge Biosciences Ltd, UK) were cast in Electro-4 Gel tanks (Thermo Fisher Scientific Waltham MA). Samples containing loading buffer were loaded alongside negative controls and DNA ladder. Agarose gels were run at 65V for ~45 minutes until fragments had migrated and DNA bands visualised under UV light using a GeneFlash Transilluminator (Syngene Bio Imaging).

3.2.2b Extraction of bacterial genomic DNA

DNA was extracted from *sej* positive *S. aureus* strain ATCC 13565 in order to amplify the *sej* gene for generation of an expression vector.

Buffers

VHBio Bacterial Genomic DNA Purification Kit

Procedure

Sterile TSB was inoculated with *Staphylococcus aureus* (ATCC 13565) and incubated overnight (37°C, 150rpm). Resulting culture was plated on agar and incubated overnight at 37°C. A colony was used to inoculate TSB, which was incubated as before for five hours. 1ml of culture was removed and pelleted. Supernatant was discarded and pellets resuspended in 100µl spheroblast buffer and 3µl lysostaphin. Samples were incubated at 37°C and 25 rpm for 10 minutes. 25µl lysis buffer was added and samples incubated in a 63°C water bath for five minutes. 25µl Advamax beads and 25µl extraction buffer were added and samples vortexed. Cell debris was pelleted (13000rpm, 3 minutes) and supernatant discarded. Pellets were washed in 70% ethanol. Supernatant was discarded and pellets left to dry for 2 hours. Pellets were resuspended in 50µl sterile water and stored at 4°C.

3.2.2c Generation of plasmids expressing recombinant SEJ

Primers

Primers were designed using the sequence of *sej* accessed via the NCBI database (AF053140). Signal peptides were predicted using SignalP software and cleavage positions revealed to be either between amino acids 24 and 25 or 25 and 26. Two coding primers with melting points of around 70°C were designed from these points in the sequence (base 72 and 75 respectively), designated primer A and primer B. BamHI (ggatcc) and HindIII (aagctt) restriction sites (shown in bold) were also designed into primers. The non-coding primer also contained a stop codon (show in italics)

Primer A

Coding

5'-ggt**ggatcc**gatagcaaaaatgaaacaattaaagaaaag–3'

Primer B

Coding

5'-ggt**ggatcc**agcaaaaatgaaacaattaaagaaaaga-3'

Non-coding (used for both)

3'-ccc**aagctt***tca*cagaaccaaaggtagacttattaata-5'

Primers were purchased from VHBio Ltd (UK) and supplied as lyophilised powders which were rehydrated in sterile TE to 100μM stocks and diluted to 5μM in dH_2O.

Promega GoTaq and GoTaq Flexi buffer were used to prepare PCR master mixes at the following concentrations (Table 3.1):

Table 3.1 Concentrations of PCR reagents

Reagent	Stock concentration	Final concentration in PCR
dH_2O	-	-
$MgCl_2$	25mM	3mM
GoTaq Flexi Buffer	5 x	1 x
dNTPS	10mM	200µM
Forward primer	5µM	0.5µM
Reverse primer	5µM	0.5µM
GoTaq	4U/µl	1U
DNA	100ng/ml	100ng

3.2.2d Expression of SEJ using pPRO-EXHTb

sej gene was amplified from genomic DNA using polymerase chain reaction (PCR) using the following conditions: 2 minutes initial denaturation at 94°C, 7 cycles of 1 minute at 94°C, 1 minute at 62-55°C (-1°C each cycle), 1 minute at 72°C, followed with 30 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C, giving a total of 37 cycles, followed by a further 10 minutes at 72°C. Size and purity of PCR product was analysed by agarose gel electrophoresis and *sej* extracted using PelletPaint® Coprecipitant (Novagen, Madison, WI) following manufacturer's instructions.

Purified gene product and pPROEX-HTb vector, coding a histidine tag and ampicillin resistance gene, were digested using BamHI and HindIII (New England Biolabs, MA). Resultant DNA was run on an agarose gel digested vector and insert extracted from gel slices using glass wool centrifugal filters and PelletPaint®. Ligations were carried out in 10µl reactions at 4°C over 72 hours using DNA T4 Ligase (New England Biolabs, MA).

Competent XL-1Blue cell were transformed with ligations by electroporation and quenched with LB broth before plating on LB agar and incubating overnight at 37°C. One colony was used to inoculate LB broth which was incubated overnight with shaking.

3.2.2e Expression of SEJ in TOPO pCR2.1 Vector (Invitrogen)

Following difficulties expressing SEJ in pPROEX-HTb attempts were made to clone *sej* into the sub-cloning vector TOPO pCR2.1. TOPO pCR2.1 vector contains poly-T overhangs for

use with PCR products amplified with *Taq,* which adds poly-A tails to the ends of sequences.

sej was amplified and purified as described above. Ligations into TOPO pCR2.1 vector were conducted according to manufacturer's instructions (Table 3.2).

Table 3.2 Ligation of sej into TOPO pCR2.1 vector

Reagent	Volume (µl)
PCR product	3
Salt Solution	-
1:4 salt solution	1
dH_2O	1
TOPO vector	1
Total	6

Ligations were left at RT for 5 minutes. Electrocompetent TOP10 cells were transformed with ligations and quenched with LB broth before incubation for 1 hour at 37°C with shaking. Cell suspensions were plated onto LB agar containing 100µg/ml ampicillin, 50µg/ml kanamycin, 0.5mM IPTG and 10% X-Gal and incubated overnight at 37°C. Plasmid DNA was extracted from positive (white) colonies using QIAprep Spin MiniPrep kits (Qiagen UK) and digested with BamHI and HindIII or EcoRI. Fragments were subjected to agarose gel electrophoresis for verification of ligation.

3.3 Results

3.3.1 Streptococcal Pyrogenic Exotoxins (SPE) A, C, L and M

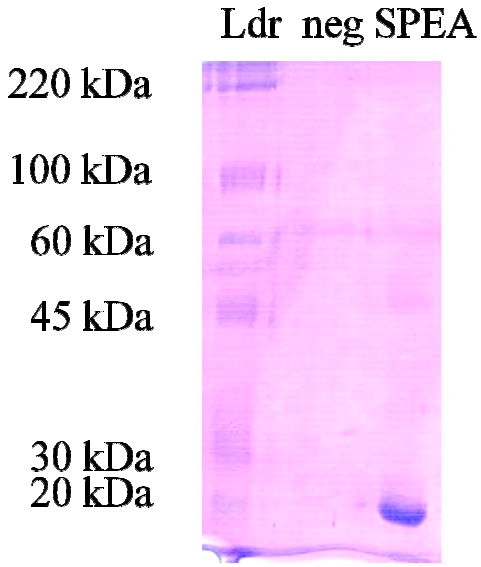
3.3.1a Expression and validation of Streptococcal Pyrogenic Exotoxin A

Expression

His-tagged SPEA, to be used as an example of a classical superantigen, was expressed from BL21 cells donated by Professor Shiranee Sriskandan (Tables 2.6 and 2.7) and purified on HisBind columns Figure 3.1 shows the resultant 26kDa protein following dialysis into 1 x PBS.

Figure 3.1 Purified and dialysed SPEA

12% polyacrylamide gel showing a single band of 26kDa corresponding to the anticipated size of his-tagged SPEA. Bands were visualised using coomassie blue stain.



Validation

To validate the biological properties of purified rSPEA I conducted an *in vitro* proliferation of PBMCs and analysed the Vβ responses by flow cytometry over a range of concentrations. For each sample >50000 events were analysed and CD4 vs Vβ staining assessed. Two different gating strategies were investigated. First all T-lymphocytes were gated on based on forward and side scatter properties (Figures 3.2ai and ii) and the percent of total CD4+ T cells calculated for each Vβ using:

$$(Q2 \div (Q2 + Q4)\,) \times 100$$

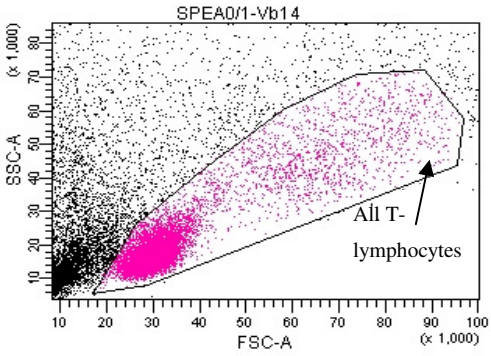
A second gating strategy utilised in similar experiments previously [66, 73] was also used to distinguish between 'resting' and 'blasting' lymphocytes. Resting and blasting T-lymphocytes were gated on based on forward scatter and side scatter properties, with approximately 2000 events falling into the blasting T-lymphocyte gate (Figures 3.2bi, ii and iii). Percents of total CD4+ T cells were calculated for each population using the above equation. Results for all three populations were then plotted as in Figures 3.3a, b and c below.

Figure 3.3 shows the Vβ repertoire in three populations of lymphocytes following stimulation with rSPEA. Figure 3.3a shows that by gating on all T-lymphocytes, whilst the proportion of Vβ2 is universally higher than other Vβs, there is no dosage response seen and no difference between rSPEA and PHA responses. Gating on resting lymphocytes (Figure 3.3b) shows a suppression of Vβ14 compared to the PHA response and a slight increase in proportion of Vβ2 with no differences observed in other Vβ types. When blasting lymphocytes are gated, at the lowest concentration used, a marked Vβ specific expansion of Vβ14 is seen, with corresponding decreases in the proportion of the total CD4+ lymphocyte populations made up by other Vβs, as would be predicted. As concentration increases, the proportion of Vβ14 decreases. This is due to expansion of other Vβ types (12, 13 and 3 not included in the panel of Vβs tested [73]. This demonstrated that in order to see the expected Vβ expansion the optimal gating strategy to use is gating on resting and blasting lymphocytes separately. This strategy was employed for all further Vβ proliferation assays described in this chapter and Chapter 6.

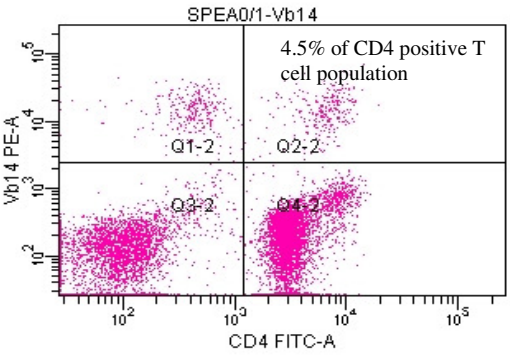
Figure 3.2 – Differing approaches to gating in Vβ proliferation assays

Showing gating on all T-lymphocytes in pink (ai and ii) and resting and blasting lymphocytes in blue and green respectively (bi, ii and iii). The percent of CD4 positive T cells bearing a specific Vβ region was calculated using (Q2/Q2+Q4))*100. These examples show that 4.5% of all lymphocytes, 0.4% of resting lymphocytes and 25.9% of CD4 positive T cells bore Vβ region 14 in response to stimulation with 0.1µg/ml SPEA.

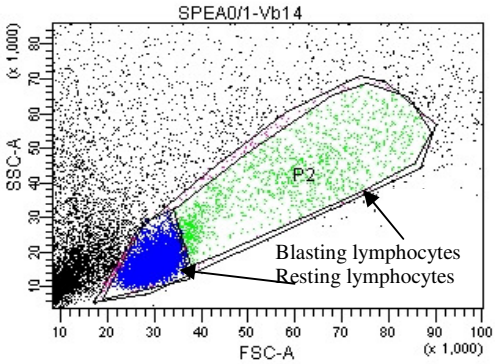
a) i



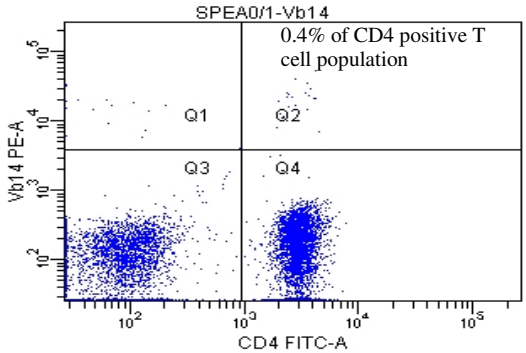
a) ii



b) i



b) ii



b) iii

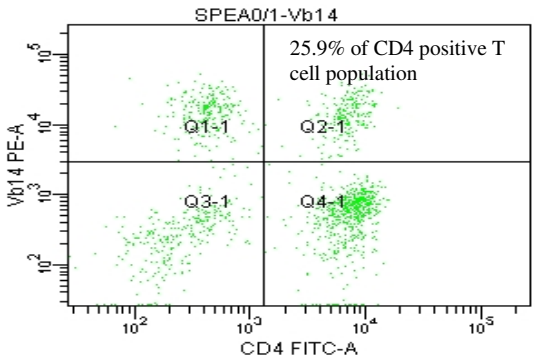
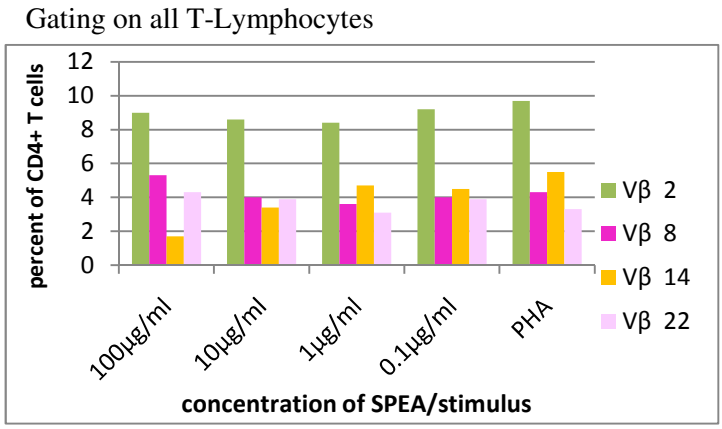


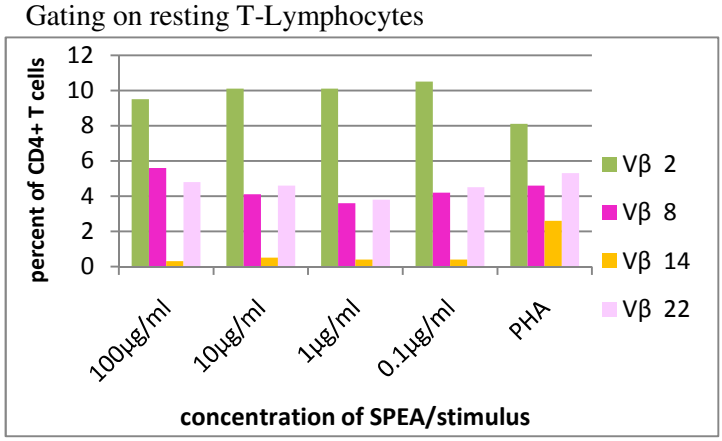
Figure 3.3 Percent of CD4+ T cells bearing specific Vβ regions in response to SPEA stimulation in different populations of T cells

Results shown are for one donor for a single experiment. Cells were stimulated with SPEA for 72hrs. The predicted Vβ expansion of SPEA is 3, 12.2, 13, and 14 [7, 73]. As concentration of rSPEA decreases, there is very little change in the percentage of CD4 positive T cell bearing any Vβ studied in either all T-lymphocytes (a) or the resting population (b) between concentrations. In the blasting population (c) as concentration of rSPEA decreases, Vβ14 as a proportion of the entire blasting population increases. Minimal expansion is observed in other Vβ's studied.

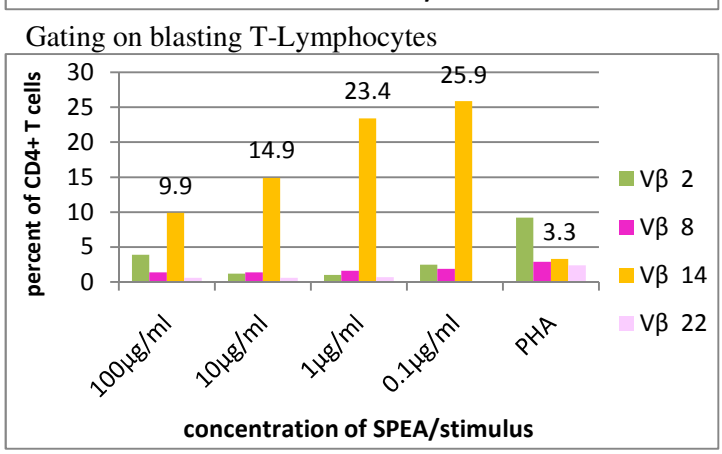
a)



b)



c)



3.3.1b Expression and validation of SPEC, L and M

Expression

IPTG induced and uninduced bacterial lysates were analysed using SDS-PAGE for the presence of a band corresponding to the correct size (Figure 3.4). A band at ~44kDa in lane 4 corresponds to protease 3C. Arrows indicate the presence of bands at sizes corresponding to SPEC, L and M (Lanes 5, 8 and 10 respectively). Toxins were purified from frozen bacterial pellets using either GSTBind Kits (SPEC) or HisBind Kits (SPEL, M).

Digestion

Initial yield of protease 3C was low and it performed poorly in digest of SPEM. Therefore a commercial protease, PreScission Protease (GE Healthcare, UK) was used. Trx tags were successfully linearised from SPEM on the first attempt (Figure 3.5a) and SPEL at the second attempt (Figure 3.5b). GST was linearised from SPEC using 1mg/ml trypsin (Figure 3.5c) following unsuccessful cleavage using PreScission Protease (Figure 3.5a). GST was removed from solution using the GSH agarose column (Figure 3.5d)

Figure 3.4 IPTG induction on expression of target gene

Following induction with 1mM IPTG for three hours Lane 4 of this 12% polyacrylamide gel shows a band of 44kDa corresponding to protease 3C. Induction of SPEM-Trx expression appears to be unclear, however bands corresponding to SPEC-GST (~46kDa) and SPEL-Trx (~48kDa) are visible in lanes 5 and 8 respectively. Bands were visualised using coomassie blue stain.

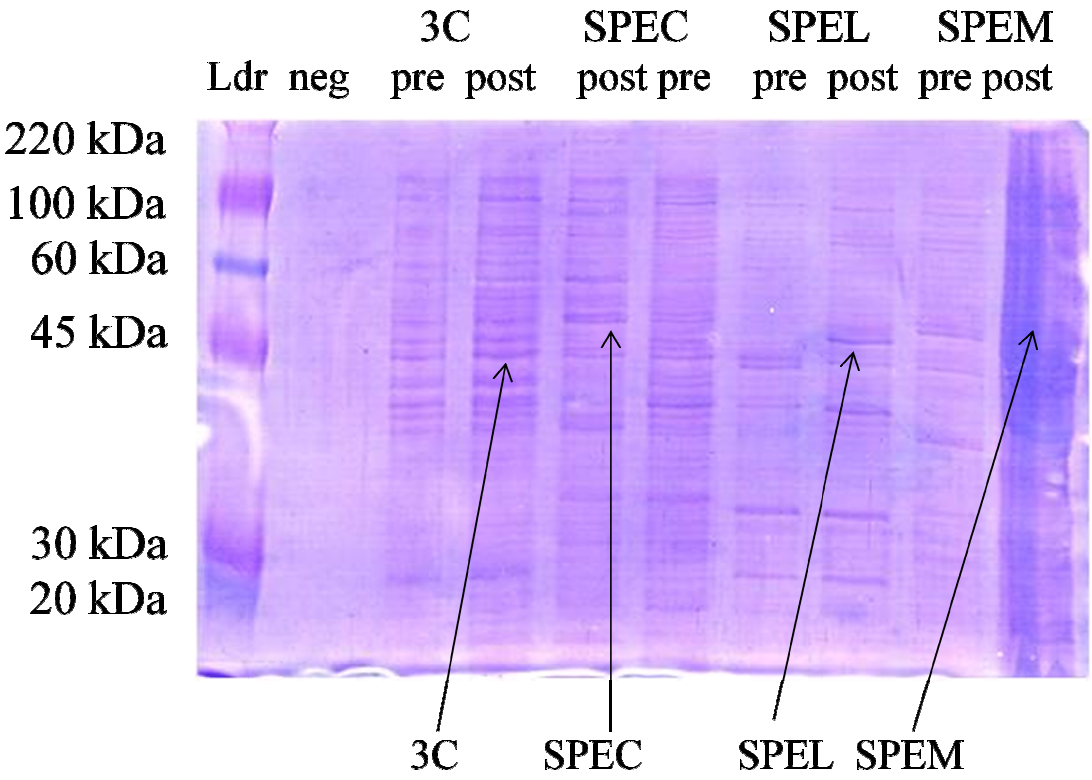
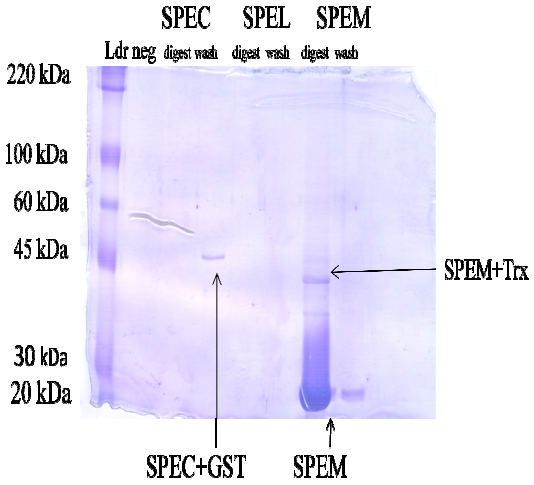


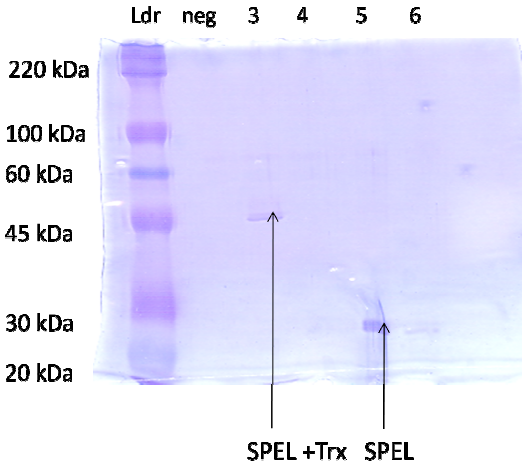
Figure 3.5 Removal of GST and Trx tags

12% polyacrylamide gels showing: (a) SDS-PAGE of linearised SPEM showing a band at ~26kDa in digest and column wash. Lane 4 shows the unsuccessful cleavage of SPEC/GST with a single band at ~44kDa. (b) Cleavage of SPEL/Trx showing a band at ~27kDa corresponding to SPEL in Lane 5 and SPEL + Trx in Lane 3 (c) Successful cleavage of SPEC/GST in solution using 1mg/ml trypsin showing a band at ~23kDa. (d) Showing fraction of SPEC before (lane 4) and after (lane 5) GST removal from solution. Bands were visualised using coomassie blue stain.

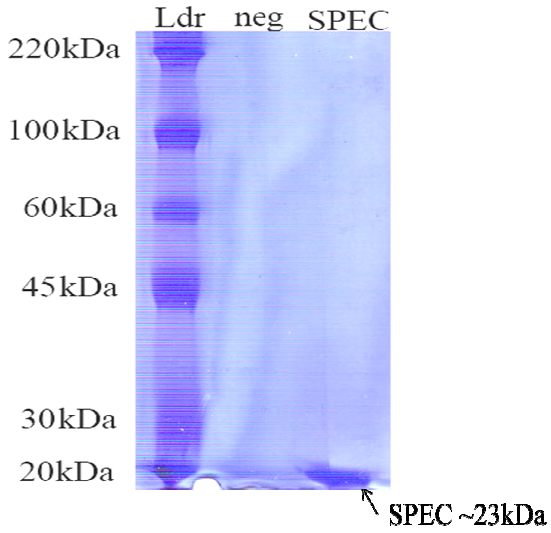
(a)



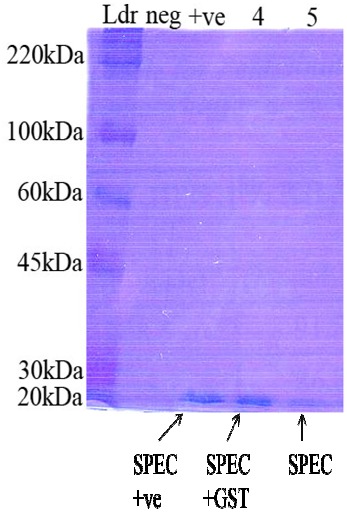
(b)



(c)



(d)



Validation of SPEC, SPEL and SPEM

Following cleavage of tags from SPEC, SPEL and SPEM, purified recombinant superantigens were analysed using a BCA protein assay and frozen in aliquots. Toxins were used in T cell proliferation assays. The Vβ signature of SPEC is documented as 2.1, 3.2, 12.5 and 15.1 [58]. Results of the SPEC validation indicate a Vβ12 specific response; however Vβ8 also expanded in a dosage respondent manner, peaking at 0.1ng/ml, whereas Vβ2 showed very little response (Figure 3.6).

A Vβ proliferation of SPEL was also carried out (Figure 3.7a). The Vβ profile of SPEL is documented as 1.1, 5.1 and 23.1 [58]. Vβ23 and Vβ5.1 expansion is seen at 10µg/ml SPEL. Vβ1 expansion is seen to a lesser extent at 1µg/ml while no significant expansion of Vβ12 is seen. Interestingly as concentration of SPEL decreases, proportions of all Vβs decreases, which may suggest another Vβ not tested here is expanded. A second Vβ proliferation was carried out using a lower range of concentrations of SPEL and the same donor (Figure 3.7b). As the concentration of SPEL decreases below the range originally used, expansions in both Vβ1 and 5.1 are seen. The percentage of Vβ1 peaks at 0.1µg/ml, whilst Vβ5.1 peaks at a concentration 1000 fold lower at 0.1ng/ml. Vβ23 expansion appears to be less important at lower concentrations of toxin.

Finally a validation was carried out using SPEM, which is documented as expanding 1.1, 5.1 and 23.1 [58]. At the lowest concentration of SPEM used there was an expansion in Vβ1 and Vβ5.1. This is seen at a lesser extent at higher concentrations, where the proportion of Vβ23 T cells increases, similar to the case with SPEL. (Figure 3.8).

Figure 3.6 Percent of CD4+ T cells bearing specific Vβ regions in response to SPEC stimulation

Results are for one donor in a single experiment following stimulation of PBMCs with SPEC for 72hrs. SPEC is documented to cause an expansion in Vβs 2.1, 3.2, 12.5 and 15.1 [7, 58]. As concentration of SPEC decreases, the percentage of CD4 positive T cell bearing Vβ2 does not change but Vβ8 appears to expand in a dosage dependent manner. Vβ12 shows expansion, however this is not dosage dependent.

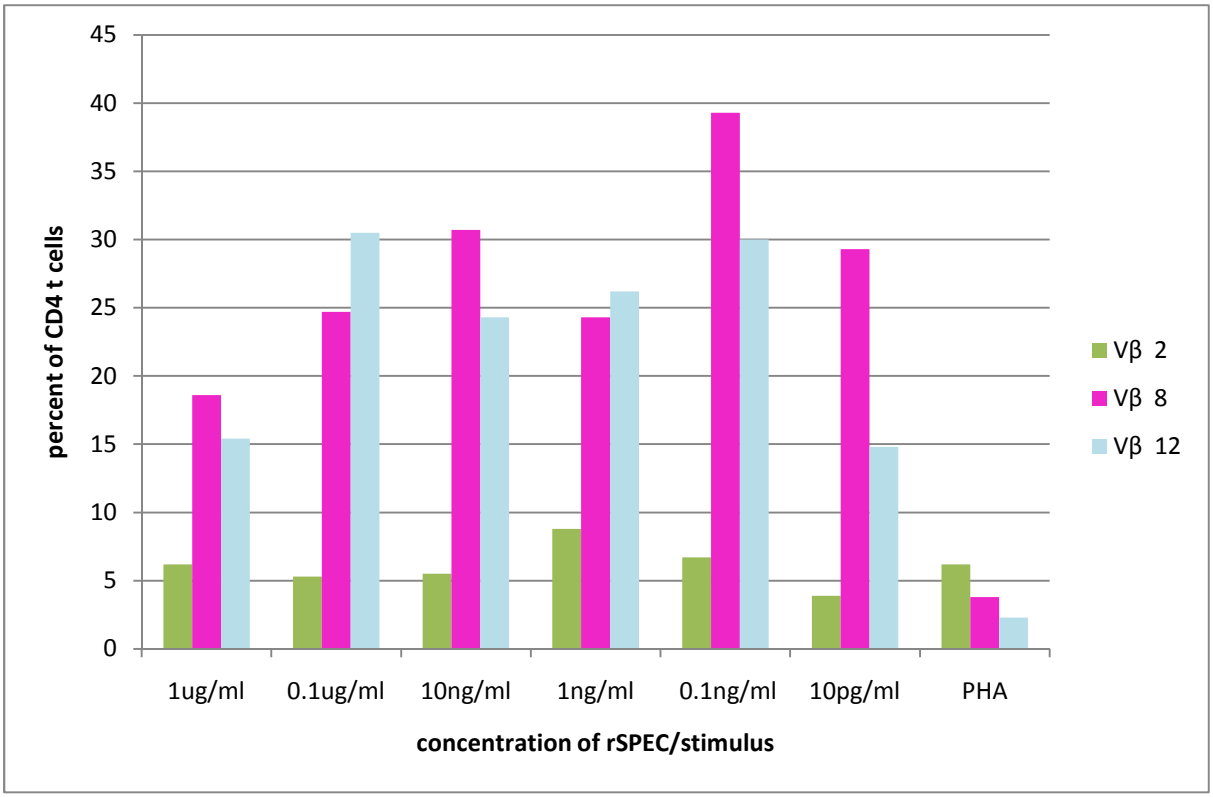


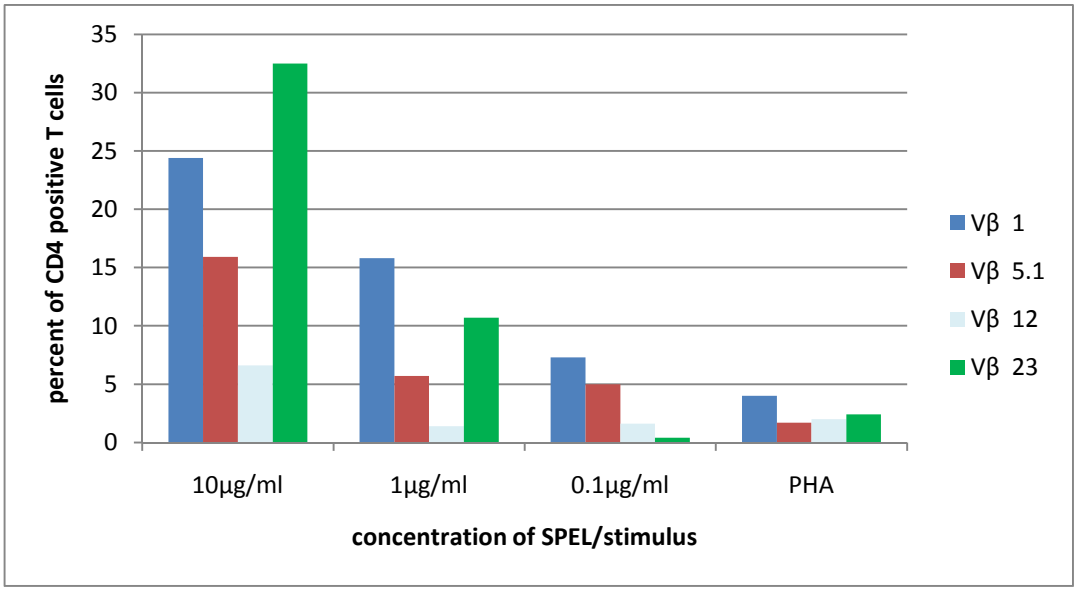
Figure 3.7 Percent of CD4+ T cells bearing specific Vβ regions in response to SPEL stimulation

Results are for one donor in a single experiment following stimulation of PBMCs with SPEL for 72hrs.

a) Expected expansion of Vβ1, 5.1 and 23 is observed. No significant expansion of non-responsive Vβ12 is seen.

b) Expected expansion in Vβs 1 and 5.1 is seen and the two peak at different concentrations. Vb23 expansion is less important at lower concentrations of SPEL than in the higher range. Vβ12 appears to be expand to a lesser extent.

a) Validation of superantigenicity



b) Responses to SPEL at lower concentrations of toxin

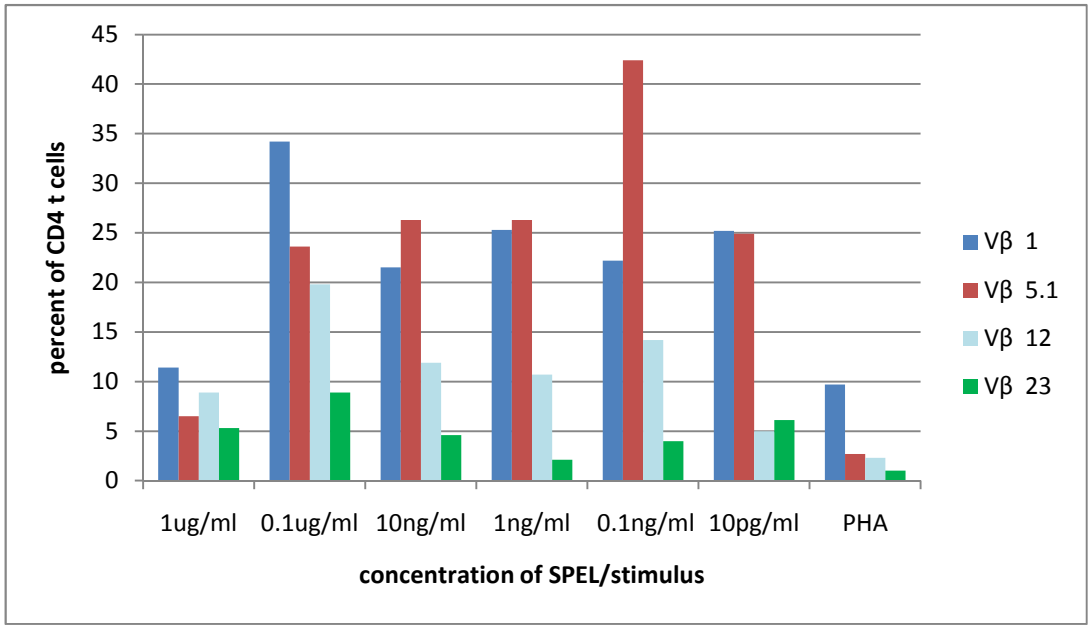
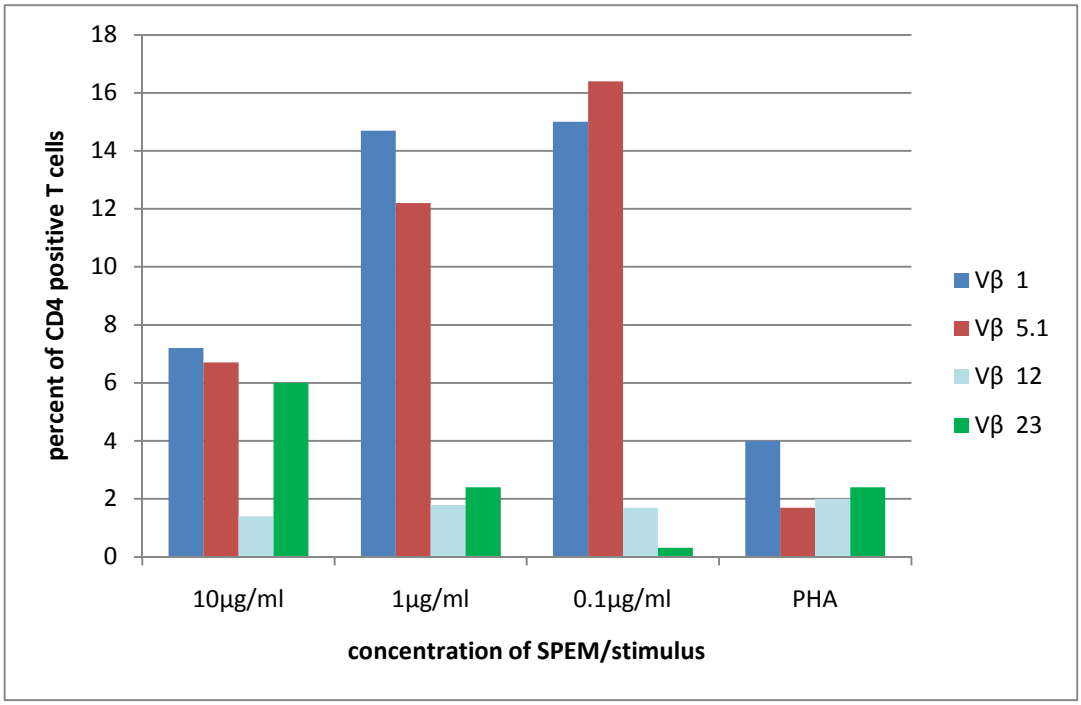


Figure 3.8 Percent of CD4+ T cells bearing specific Vβ regions in response to SPEM stimulation

Results are for one donor in a single experiment following stimulation of PBMCs with SPEM for 72hrs. SPEM is documented to cause an expansion in Vβs 1, 5.5 and 23 [7]. As concentration of SPEM decreases, the percentage of CD4 positive T cell bearing Vβ1 and Vβ5.1 as a proportion of the entire blasting population increases. An expansion of Vβ23 is seen at higher concentrations of SPEM. Minimal expansion is observed in Vβ9 and Vβ12



Following an initial validation of the superantigenicity of SPEM, a further T cell proliferation was carried out using an extended range of concentrations and the same donor (Figure 3.9). The proportion of Vβ1 increases with decreasing concentration of SPEM, and interestingly Vβ23 also appears to expand at lower concentrations, in contrast to the previous experiment. Up to 50% of CD4 positive T cells are Vβ1 positive at concentrations as low as 50fg/ml. Minimal expansion is seen in Vβ5.1 or Vβ12.

Following this result, an additional SPEM Vβ proliferation was conducted using 3 donors (Figure 3.10a-c). Donor 3 in this case was the same as for previous Vβ proliferations described.

Donor 1 shows an increasing proportion of Vβ1 with decreasing concentration, and the opposite for Vβs 5.1 and 23. Minimal expansion is seen in Vβ12. Donor 2 shows the same response in terms of Vβ1; however this was not in a dosage respondent manner and remains at a constant high level across the range of concentrations. Minimal expansion is seen in all other Vβs studied. Donor 3 shows a different trend again, with the proportion of Vβ1 decreasing with decreasing concentration and Vβs 5.1 and 23 increasing with decreasing concentration...

When these three experiments are averaged some general trends can be observed (Figure 3.10d). A consistent expansion in Vβ 1 positive CD4 T cells up to 44.4% of the total CD4 positive blasting T cell population is seen. An expansion in Vβ5.1 was far more pronounced in one donor than two others, giving an overall expansion up to 18.4% at higher concentrations of superantigen. The same is true of Vβ23 showing an expansion of up to 15.3% at higher concentrations of SPEM. The difference in response between donors is possibly due to the HLA class II restrictions previously described by Llewelyn *et al* for other superantigens [73]. Further work along this line including variations between known HLA class II types are described in Chapter 6 below

Figure 3.9 - Percent of CD4+ T cells bearing specific Vβ regions in response to SPEM stimulation using a wide range of concentrations

Results are for one donor in a single experiment following stimulation of PBMCs with SPEM for 72hrs. Expected expansion in Vβs 1 and 23 is seen down to 50fg/ml. Minimal expansion is seen in Vβs 5.1 and 12.

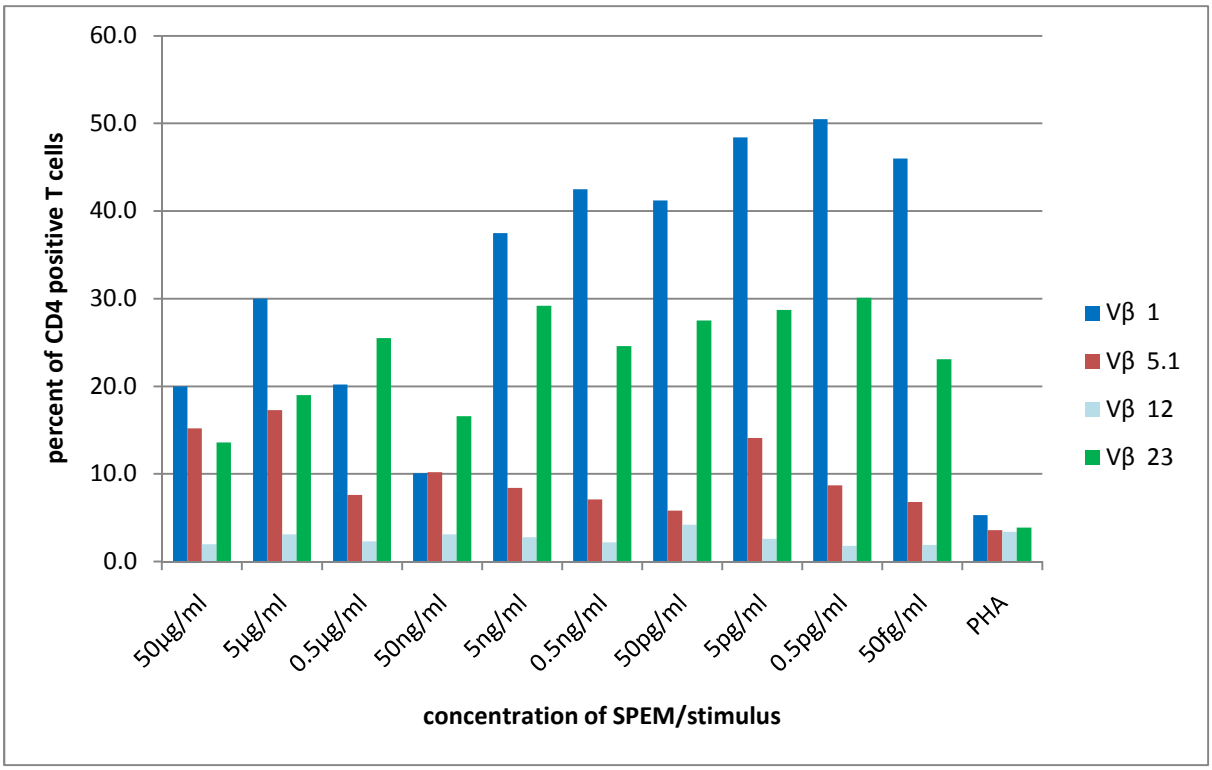
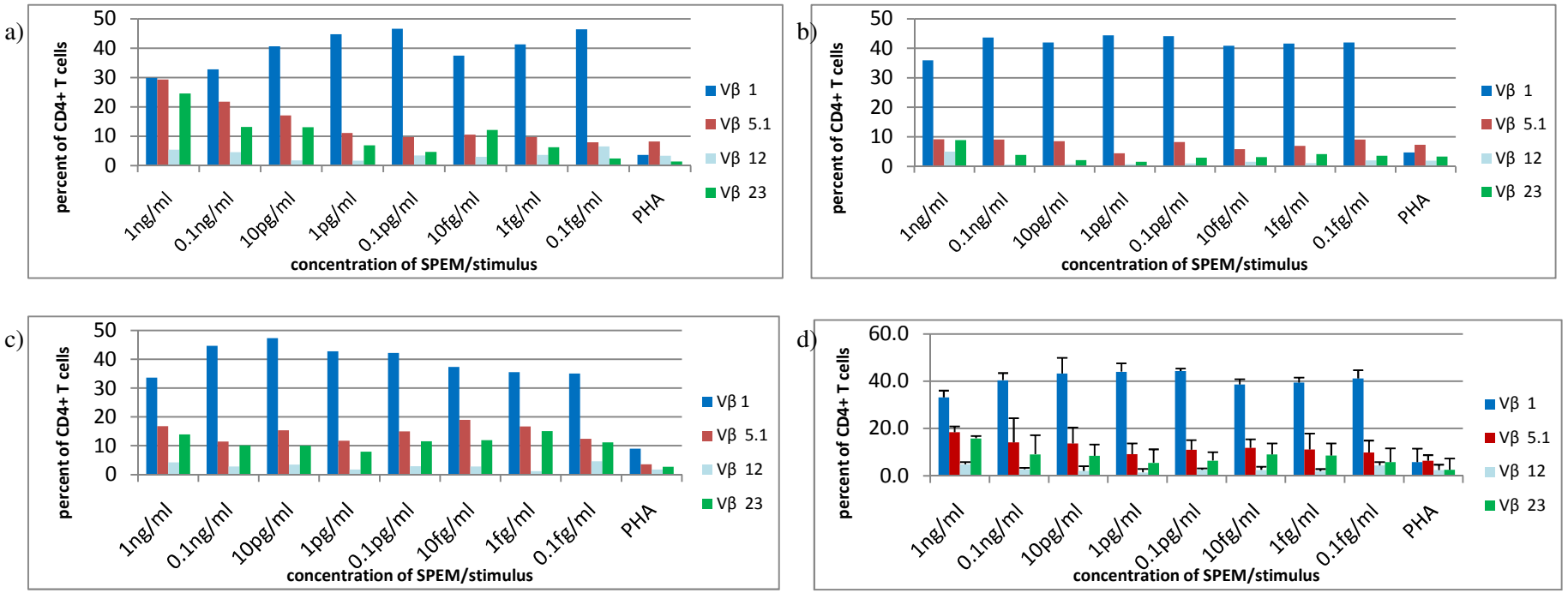


Figure 3.10 Percent of CD4+ T cells bearing specific Vβ regions in response to SPEM stimulation in three donors

Following stimulation of PBMCs from three donors with SPEM for 72hrs, results shown are for a single experiment for each donor. a) Donor 1 – Vβ1 increases with decreasing concentrations, Vβ5.1 and 23 decrease with decreasing concentrations. b) Donor 2 – Showing a consistently high proportion of Vβ1 with no dosage response and minimal expansion in all other Vβs studied. c) Donor 3 – Showing decreasing proportions of Vβ1 with decreasing concentrations and increasing proportions of Vβ5.1 and 23 with decreasing concentrations. d) Average of 3 donors. Points shown are the mean percentage of three donors plus one standard deviation. Vβ1 positive CD4 T cells form up to 44.4% of the total CD4 positive blasting T cell population. An expansion in Vβ5.1 is seen to a lesser extent up to 18.4% at higher concentrations of superantigen, and with Vβ23 up to 15.3% at the same concentration of SPEM. No expansion of Vβ12 was observed.



Following successful expression of SPEA, SPEL and SPEM the construction of a plasmid for the expression of a novel superantigen, SEJ, was attempted using an *sej* positive strain (ATCC 13565) as a starting point. Two approaches were used for this work:

- Expression in pPRO-EXHTb (Invitrogen)
- Expression in TOPO pCR2.1 (Invitrogen)

The methods used in these investigations are described above, and results described below.

3.3.2 – Generation of plasmids encoding SEJ

3.3.2a Expression of SEJ in pPRO-EXHTb

Optimisation of PCR

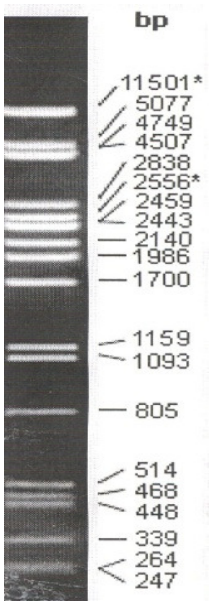
The quality of genomic DNA was assessed using an agarose gel (Figure 3.11a). Initial samples were analysed against the fragments resulting from a digest of Lambda phage DNA with Pst1 (Figure 3.11b).

sej gene was amplified from genomic bacterial DNA by PCR using primers detailed above. An initial optimisation of PCR using a temperature gradient indicated that an annealing temperature of between 55°C and 57°C, and 3mM $MgCl_2$ produced optimal results (Figure 3.12). This experiment used 30 cycles of PCR, however as the bands observed were not very bright 7 cycles were added with a temperature gradient from 62°C and 55°C (dropping by 1°C per cycle) to increase product exponentially and maximise primer binding. 30 subsequent cycles used an annealing temperature of 55°C to minimise non-specific amplification. A further optimisation using these conditions and 3mM $MgCl_2$ indicated that primer set B, 3mM $MgCl_2$ and 37 cycles of 1 minute each produced the most efficient amplification of *sej* (~800bp, Figure 3.13). Following this all further PCR reactions used primer set B and 3mM $MgCl_2$.

Consistently two fragments were amplified under these conditions (Figures 3.12 and 3.13). To rule out any possibility of amplification of two different genes due to non-specific primers the smaller product was verified as a truncated gene product (TGP) by amplification of both bands in sterile PCR master mixes, in duplicate (designated sej(a), sej(b), TGP(a) and TGP(b)). The larger band corresponding to *sej* amplified itself and the smaller product. The smaller product did not amplify, indicating non-specific binding of primers partway through the gene of interest (Figure 3.14).

Figure 3.11 – Genomic DNA from S. aureus *ATCC 13565*

a) Expected fragments of PstI digest of Lambda DNA



[174]

b) Demonstration of genomic DNA ~12000bp. In each experiment using the above DNA ladder, it was not possible to clearly resolve the individual fragments. The two bands of 1159bp and 1093bp were used as a reference point in most cases whilst other fragments are given as a range of sizes.

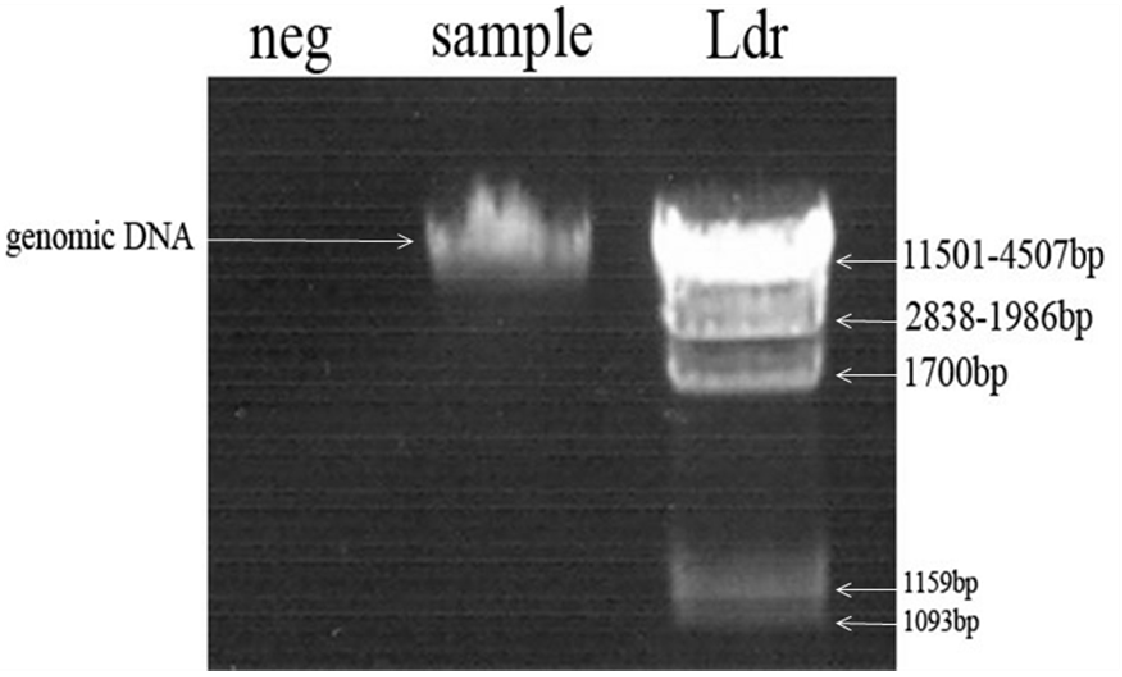


Figure 3.12 – Initial PCR optimisation

Showing faint bands present at 2.5mM and 3mM $MgCl_2$ at annealing temperatures of 55.2°C and 56.7°C, using both primers A and B. The ladder was again difficult to resolve.

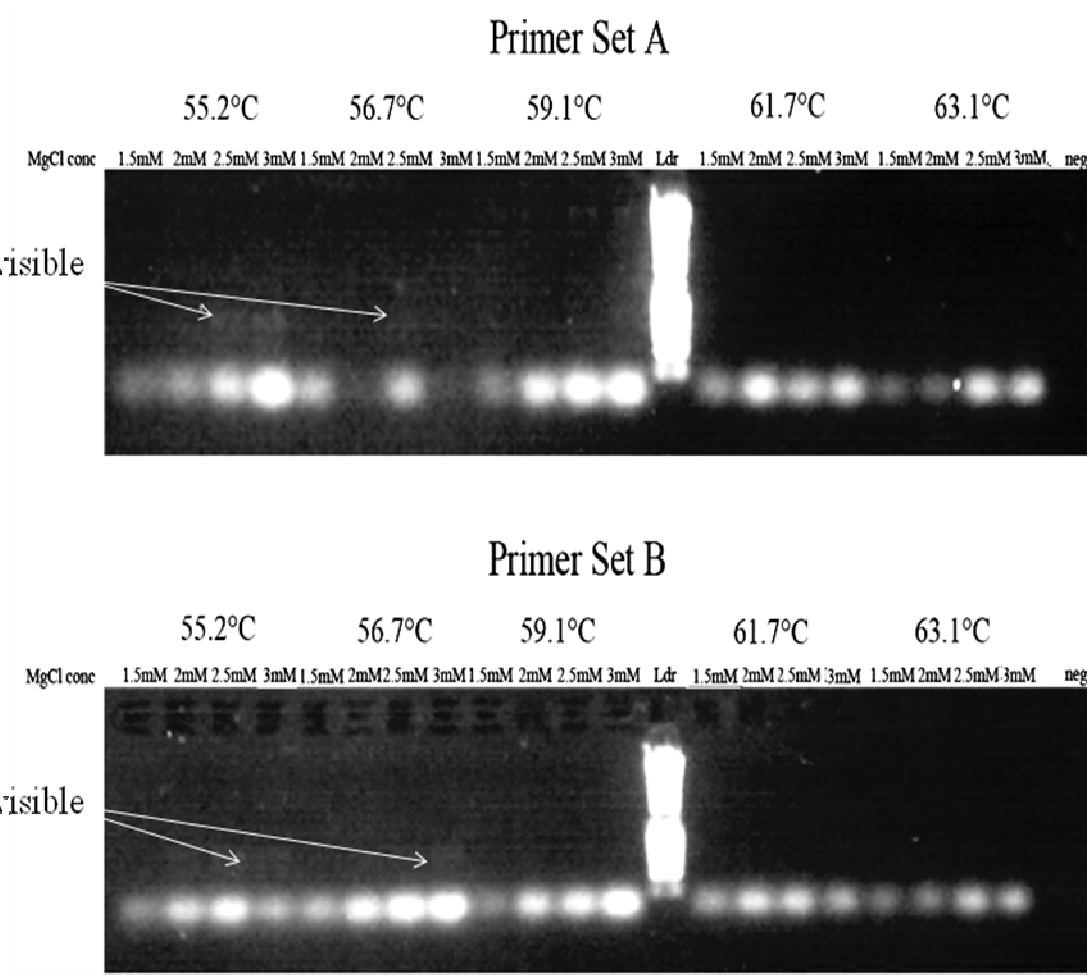


Figure 3.13- Second optimisation

Showing a brighter band at 3mM $MgCl_2$ using Primer Set B and an annealing temperature of 55°C. Two fragments are observed in every successful amplification

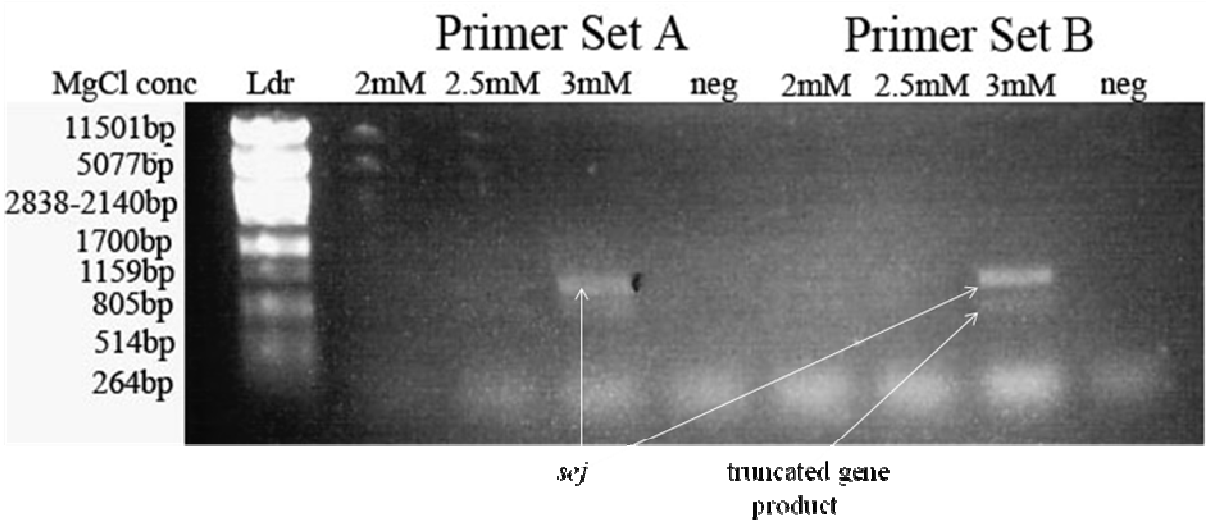
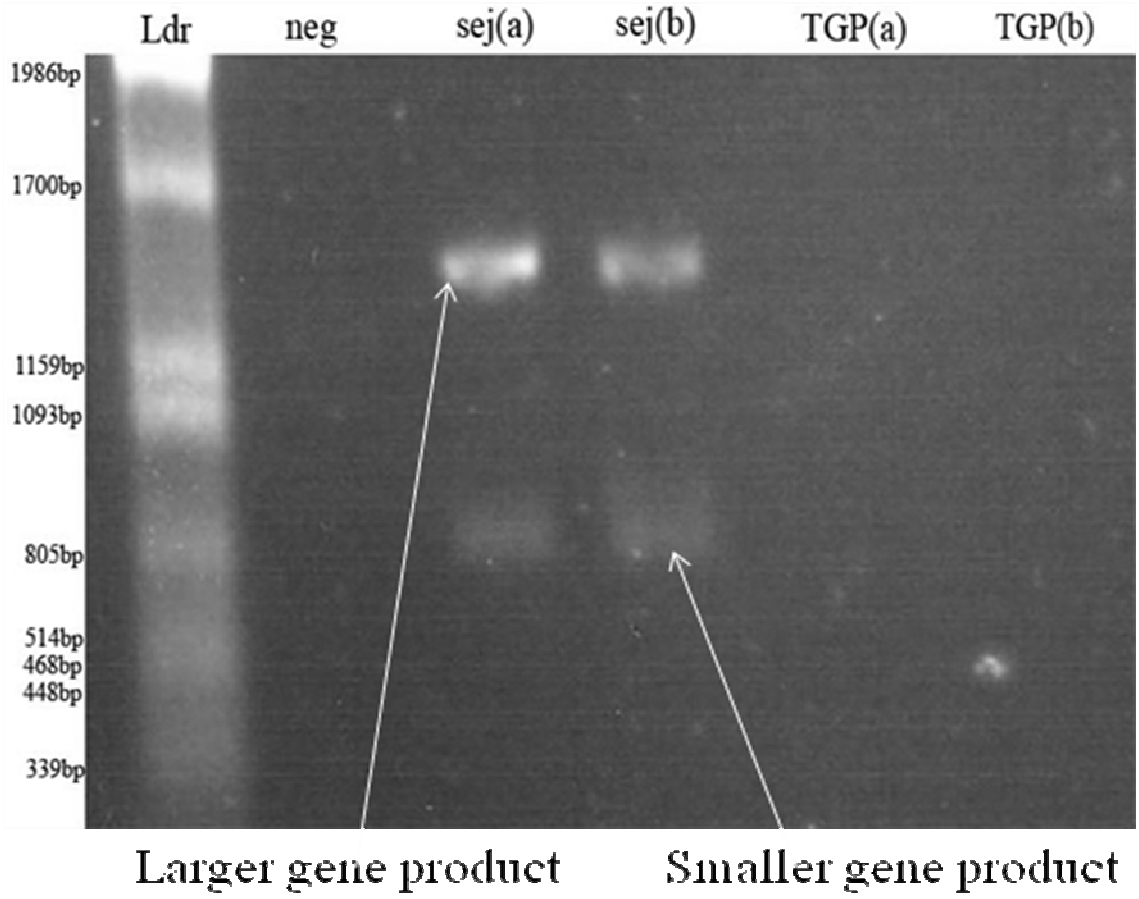


Figure 3.14 – Verification of truncated gene product

Showing the amplification of both the larger and smaller fragments from sej a + b and no amplification from TGP a + b

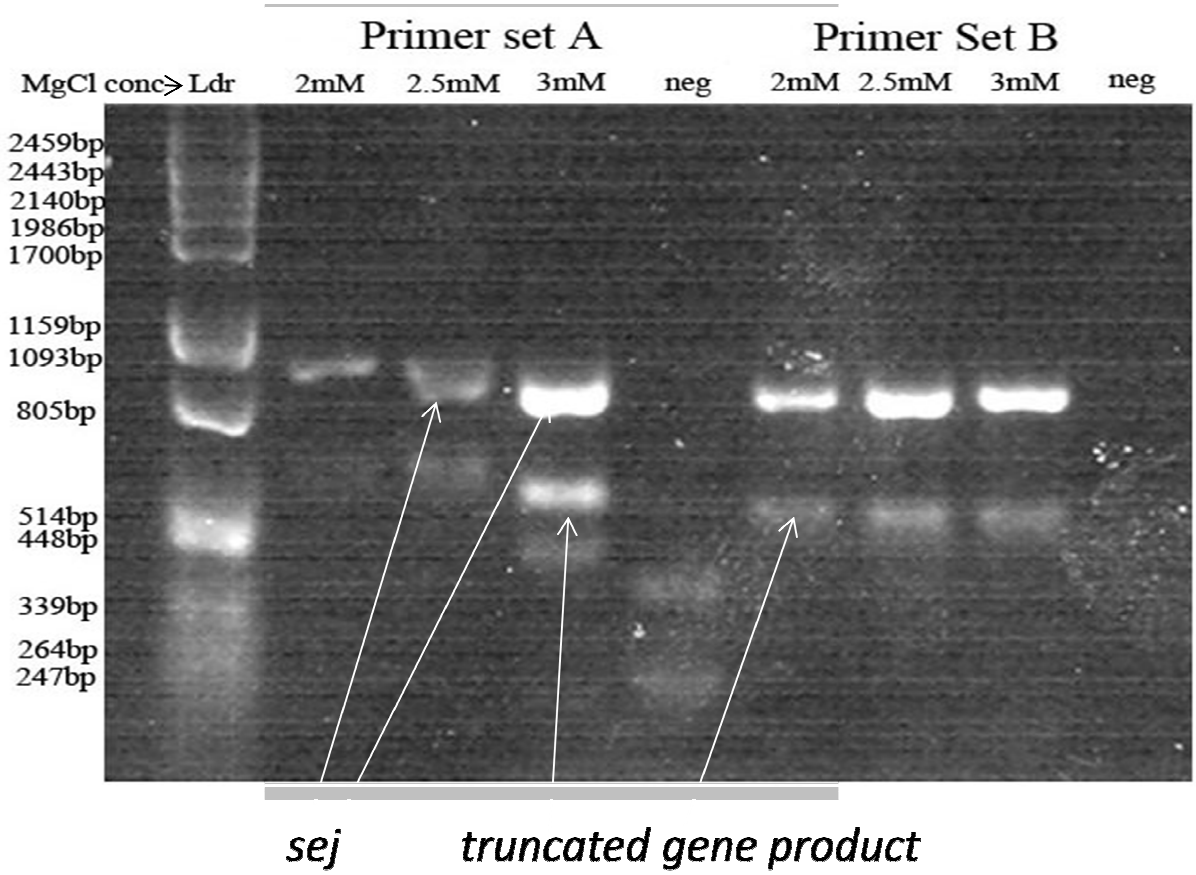


Whilst using the Lambda DNA ladder there were concerns that bands were larger than expected, although running the gel more slowly improved separation of the ladder and resolution of fragment size (Figure 3.15). This gel indicated fragments of around ~800bp and ~600bp (expected size of *sej* ~800bp). Subsequently a different DNA ladder was used for agarose gels (HyperLadder IV 100-1000bp ladder, Bioline, Taunton, MA, Figure 3.16)

Figure 3.15 – Resolution of ladder and fragment size

Showing two fragments present in each well at around ~800bp and ~600bp respectively.

The brightest bands are observed at 3mM $MgCl_2$



sej Amplification and digestion

Following optimisation of PCR conditions, the *sej* gene was amplified in large scale. An initial amplification in a 50µl reaction, using the conditions described above, resulted in no *sej*. It was thought that this was a result of a large PCR volume, therefore to determine if this gene is particularly sensitive to PCR volumes, the experiment was repeated using 5 x 10µl reactions. A lower PCR volume resulted in a successful amplification in all reactions, thus this volume was used for all future amplification reactions. The larger fragment was excised from the gel and purified using centrifugal glass wool filters and PelletPaint, however due to concerns over loss of product and purity from gel purification, a duplicate amplification was carried out and a small aliquot checked on a gel against a Hyperladder IV (Figure 3.16). This amplification and all subsequent amplifications using the same PCR volume were successful. Remaining gene product was purified from PCR product using PelletPaint CoPrecipitant (Novagen, Gibbstown NJ). An agarose gel and HyperLadder IV were used to approximately quantify gene product (Figure 3.17). Each fragment present in the HyperLadder IV corresponds to a different specific quantity of DNA. For example, in 5µl of ladder, there is 100ng of 1000bp DNA and 50ng of 500µl DNA. The brightness of the ladder bands can therefore be compared to the brightness of gene product to estimate quantity. In this case, the brightness of the band corresponding to the gene product was approximately equal to the 200bp band on the ladder, corresponding to ~20ng DNA in 5µl gene product. This could be extrapolated to indicate ~200ng DNA in the total 50µl of gene product.

Following purification of *sej* using PelletPaint, both pPROEX-HTb vector and insert were digested with BamHI and HindIII (Figure 3.18). Digests were run on a gel and bands excised. DNA was purified from the gel slices as described in the methods for this chapter.

Figure 3.16 – Amplification of sej *(iii)*

Showing a successful amplification of a gene product corresponding to the expected size of *sej* at around 800bp.

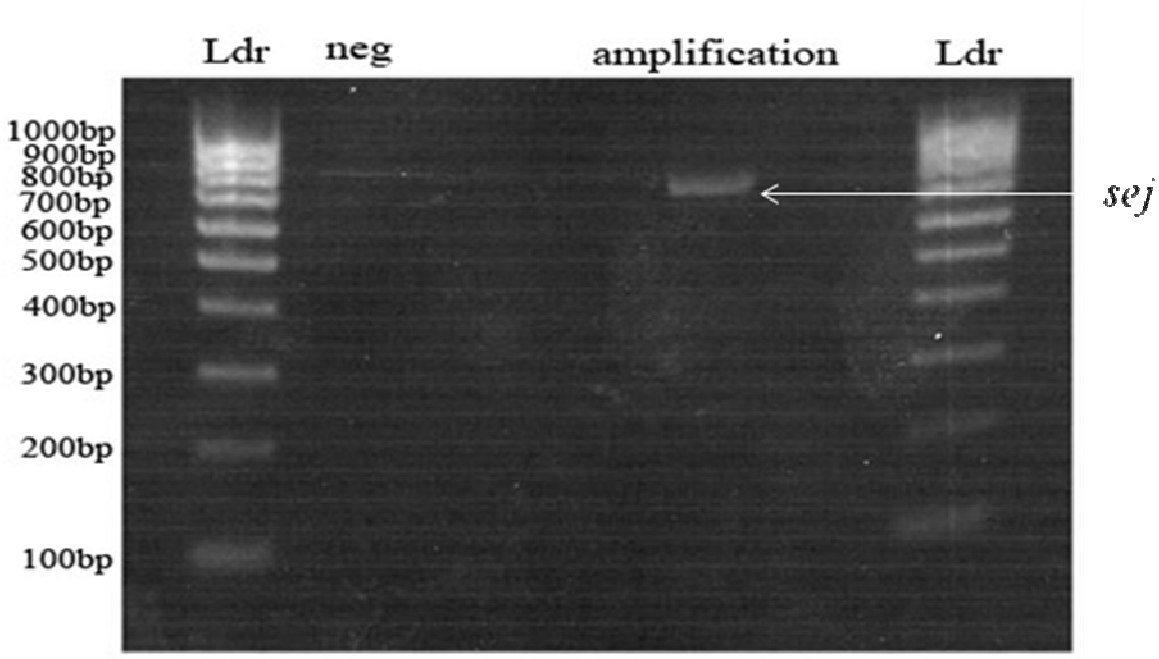


Figure 3.17 - Quantification of sej

Showing a band corresponding to approximately 20ng/5µl giving a total of ~ 200ng/50µl. Quantities of DNA present in HyperLadder IV are listed in Appendix (2). Gene product band brightness corresponds to the 200bp fragment in the ladder. 5µl of the ladder contains 20ng of 200bp DNA.

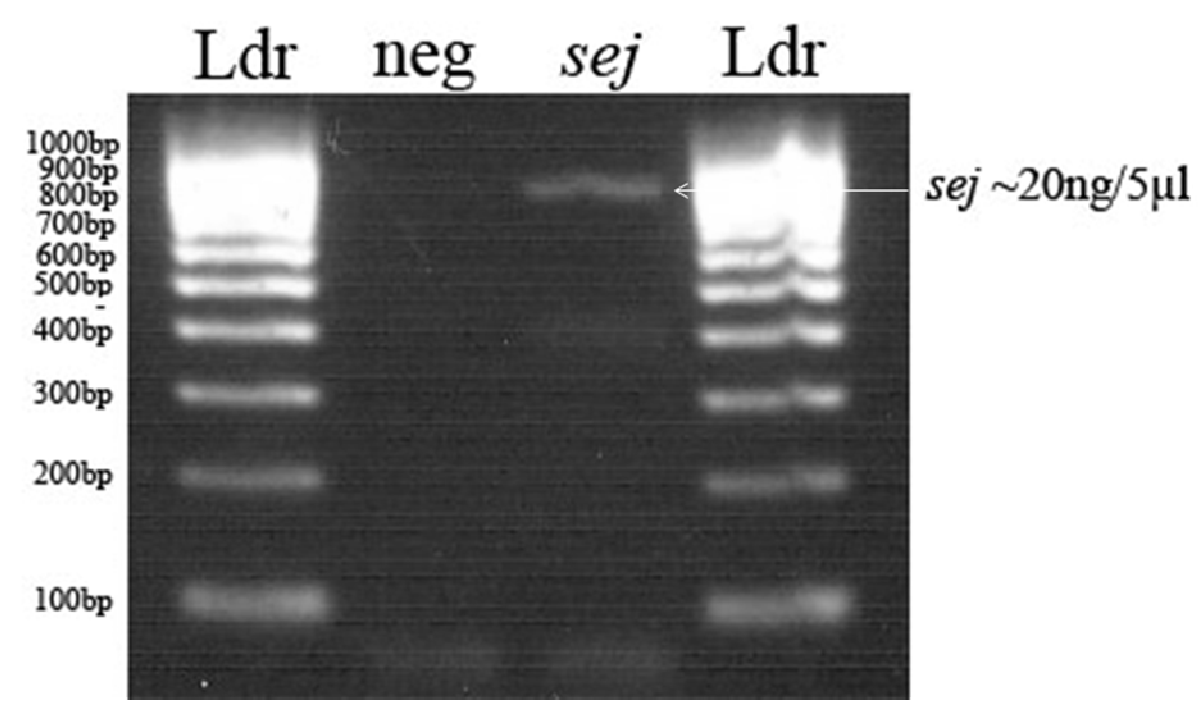
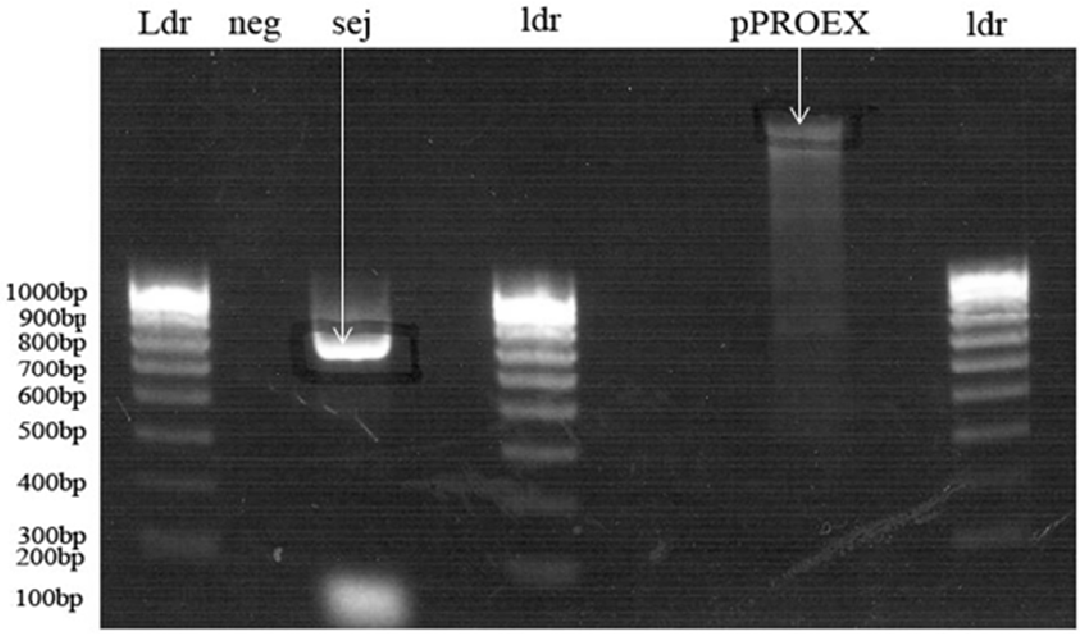


Figure 3.18 – BamHI and HindIII digest of sej *and vector*

Bands corresponding to digested *sej* and pPROEX-HTb were excised, as indicated, under UV light and gel purified.



Transformation of bacteria

Using a ratio of 1:1 and 5:1 of insert:vector based on quantitation from the gel, ligations were performed and transformed into DH5α cells using electroporation. However no colonies resulted from initial transformations therefore the process was repeated. Again no colonies were observed, therefore a ratio of 9:1 and XL1-blue cells were used. A positive control of undigested pPROEX-HTb was also used. The positive control resulted in colonies, whilst the ligation resulted in very small colonies, however overnight cultures inoculated with these colonies did not grow. Following a second unsuccessful attempt at ligation at this ratio it was decided that an alternative vector should be used for cloning, and the insert sub-cloned into pPROEX-HTb for expression.

3.3.2b Expression of sej in TOPO pCR2.1

PCR product was ligated into the TOPO vector according to manufacturer's instructions. Transformations were plated and incubated overnight at 37°C on X-gal plates and colonies resulted on all plates (Figure 3.19 for an example). Positive colonies were selected using blue-white screening, where white colonies are positive. Initially 5 positive colonies were grown overnight and QIAprep Spin MiniPrep kits were used to extract plasmid DNA (designated colonies 1-5a). Plasmid DNA was subjected to restriction enzyme digest and agarose gel electrophoresis (Figures 3.20 a and b). The expected size of the TOPO pCR2.1 vector is 3.9kb [175]. Based on this, four out of five colonies appeared to contain only linearised plasmid and uncut plasmid, however one indicated uptake of a fragment of ~1000bp (colony 2). There was a difference in band pattern in colonies 2a, 3a, 4a and 5a, which was probably attributable to differing quantities of DNA in mini-preps. Colonies 3a and 5a show a greater concentration of DNA, and the greater brightness intensity indicates some un-linearised plasmid.

TOPO pCR2.1 contains an EcoRI site either side of the multiple cloning site, therefore to determine if the ~1000bp fragment seen in colony 2 was *sej* plus a small amount of flanking sequence, an EcoRI digest of colony 2's plasmid DNA was carried out and analysed on a gel. However this proved inconclusive showing only linearised plasmid (Figure 3.21). The plasmid containing the 1000bp fragment was sent to CoGenics (Morrisville, NC) for sequencing however sequencing failed due to insufficient DNA quality.

Figure 3.19 – TOPO transformations

LB agar plate containing 100µg/ml ampicillin, 0.5mM IPTG and 10% X-gal showing a very high number of positive (white) colonies and one negative (blue) colony following ligation of insert into TOPO-pCR2.1 vector

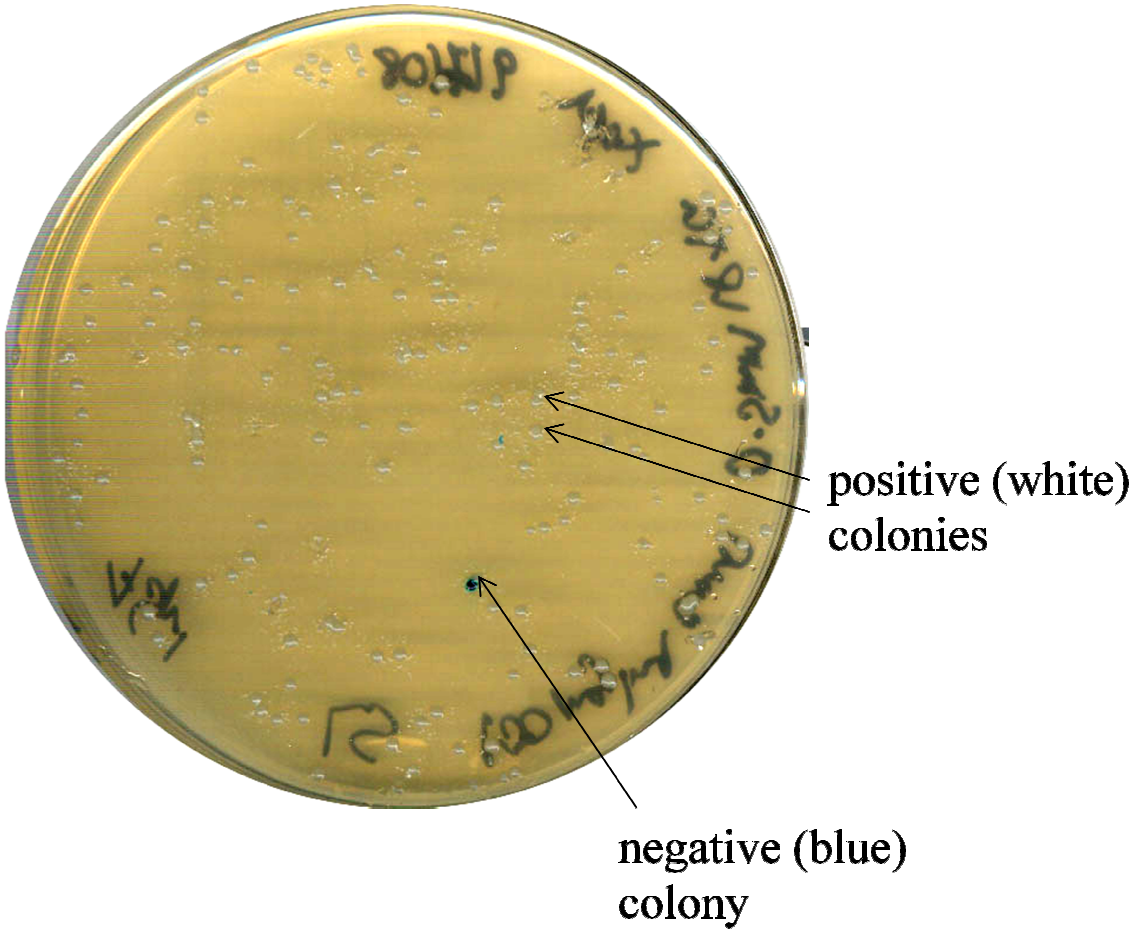


Figure 3.20a - Digest of plasmid DNA from TOPO colony 1

Restriction enzyme digest of plasmid DNA from colony 1a showing uncut and linearised plasmid and no visible insert

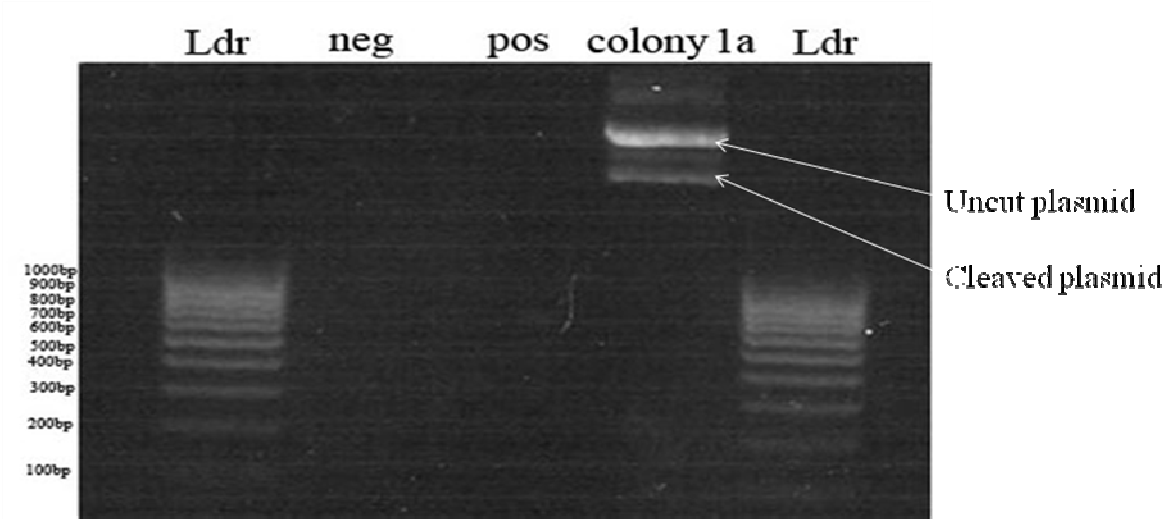


Figure 3.20b – Digest of plasmid DNA from TOPO colonies 2-5

Showing uncut and linearised plasmid in colonies 3-5a and a fragment of around 1000bp in colony 2a. The increased brightness of bands in colonies 3a and 5a is likely to be due to an excess of uncut plasmid DNA.

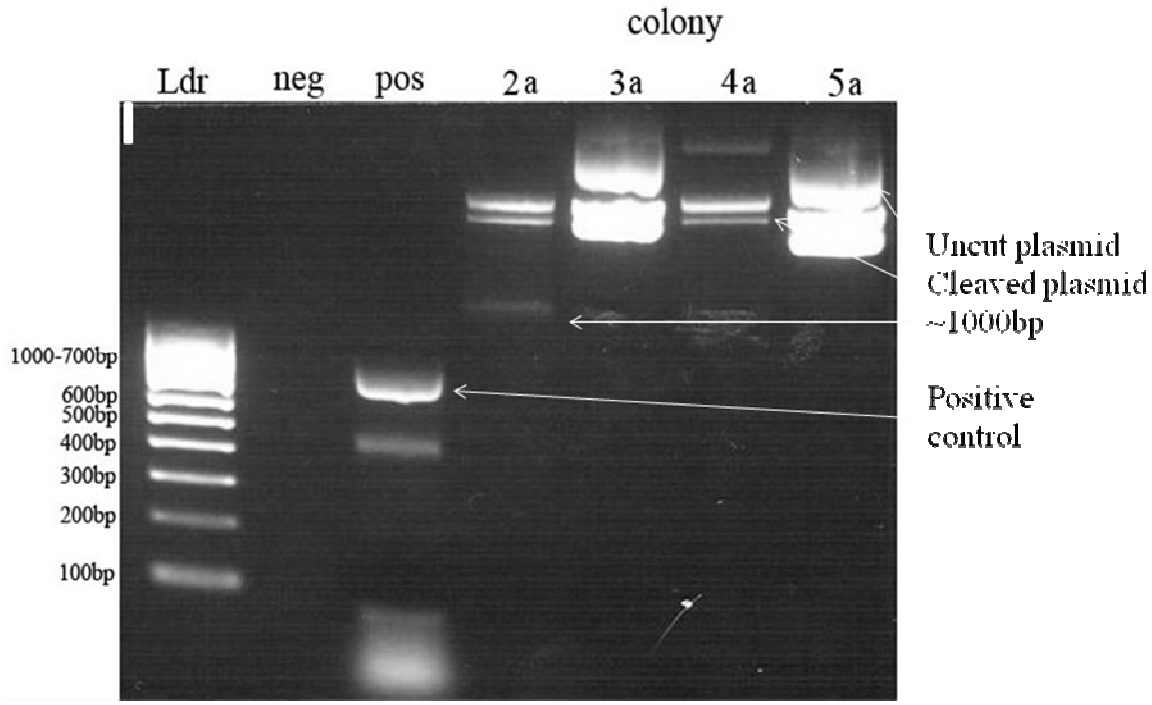
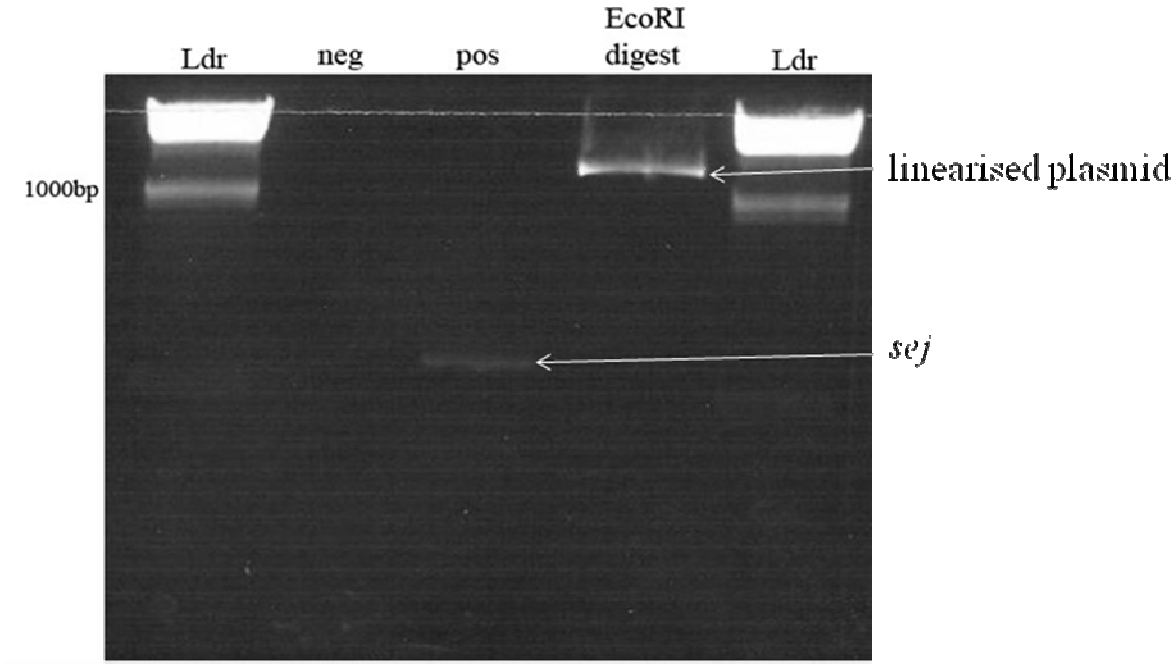


Figure 3.21 – EcoRI digest of 'Colony 2' plasmid DNA

Showing no bands following an EcoRI digest of colony 2a's plasmid DNA.



Following this 10 more colonies from the initial TOPO ligation were screened in the same manner (Figure 3.22, colonies 11-20a). This screen indicated a combination of just linearised plasmid (colonies 11a, 12a and 16a), uptake of the 1000bp fragment (colonies 13a, 14a and 20a) and uptake of the truncated gene product (colonies 15a, 17a, 18a and 19a). Samples of colonies 13a and 19a were sent for sequencing but again sequencing failed. An EcoRI digest was also performed on these colonies (Figure 3.23) which indicated that the plasmid had been cut into fragments of various sizes, however all fragment sizes were much larger than expected. Therefore, due to sequencing problems, inconsistent EcoRI results, and to eliminate the possibility of uptake of truncated gene product a second TOPO ligation was performed. Insert for this ligation was generated by PCR conducted under the optimal conditions described above and product purified by gel excision of the larger product and PelletPaint on the eluent.

Second ligations also produced colonies which were screened in the same manner. 10 colonies all indicated the ligation of the >1000bp fragment (Figure 3.24, colonies 1-10b) however the concentration of DNA was not sufficient for sequencing. A further 10 colonies from this ligation were screened, again revealing universal uptake of the >1000bp fragment (Figure 3.25, colonies 11-20b). These samples were pooled and sent for sequencing which unfortunately failed once more.

Figure 3.22 – Screen of 10 further colonies from initial TOPO ligation

Showing religated plasmid (colonies 1, 2 and 6), truncated gene product (colonies 5, 7, 8 and 9) and 1000bp fragment (colonies 3, 4 and 10)

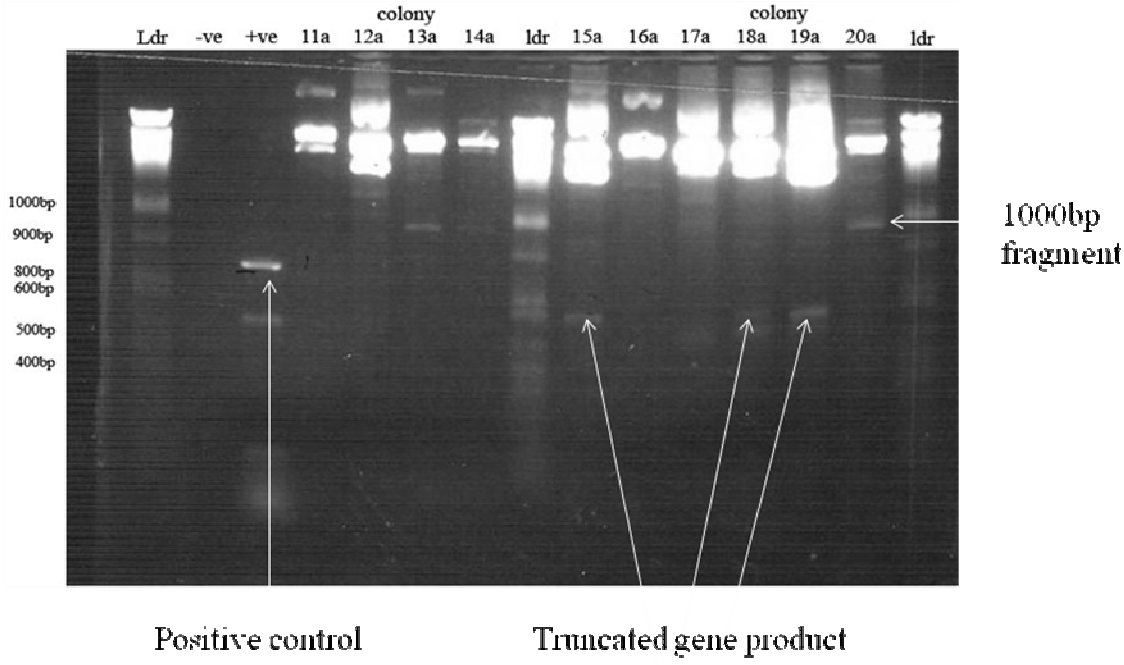


Figure 3.23 - EcoRI digest of 10 TOPO colonies

Showing digestion of plasmid into fragments of various size, all larger than expected size of *sej* of ~800bp.

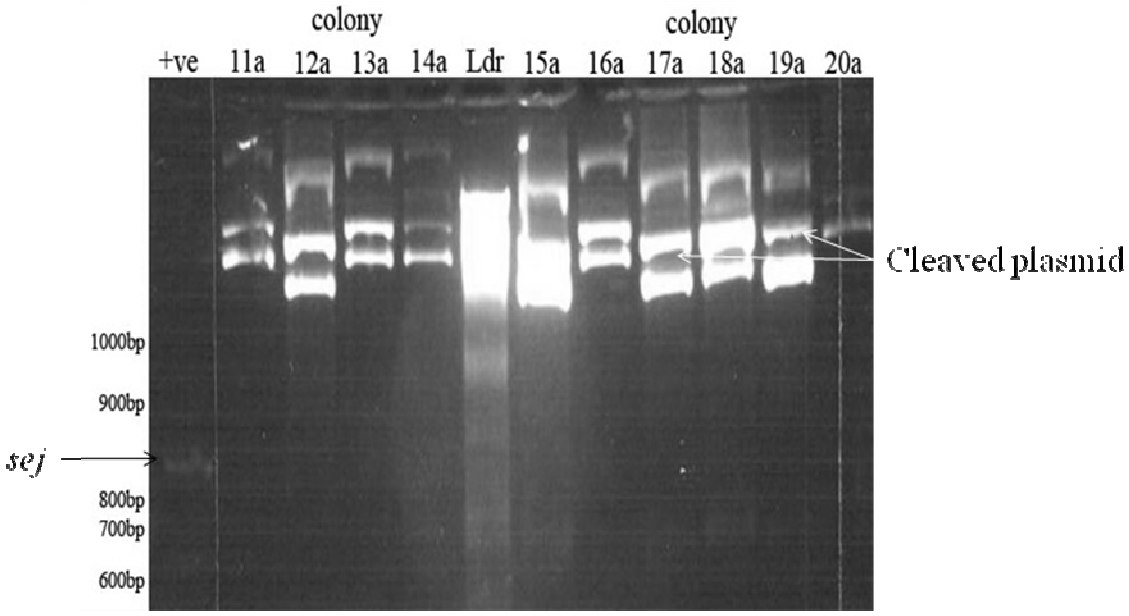


Figure 3.24– Second TOPO transformation (first 10 colonies)

Showing a universal uptake of >1000bp fragment, with the brightest band present in colony 9

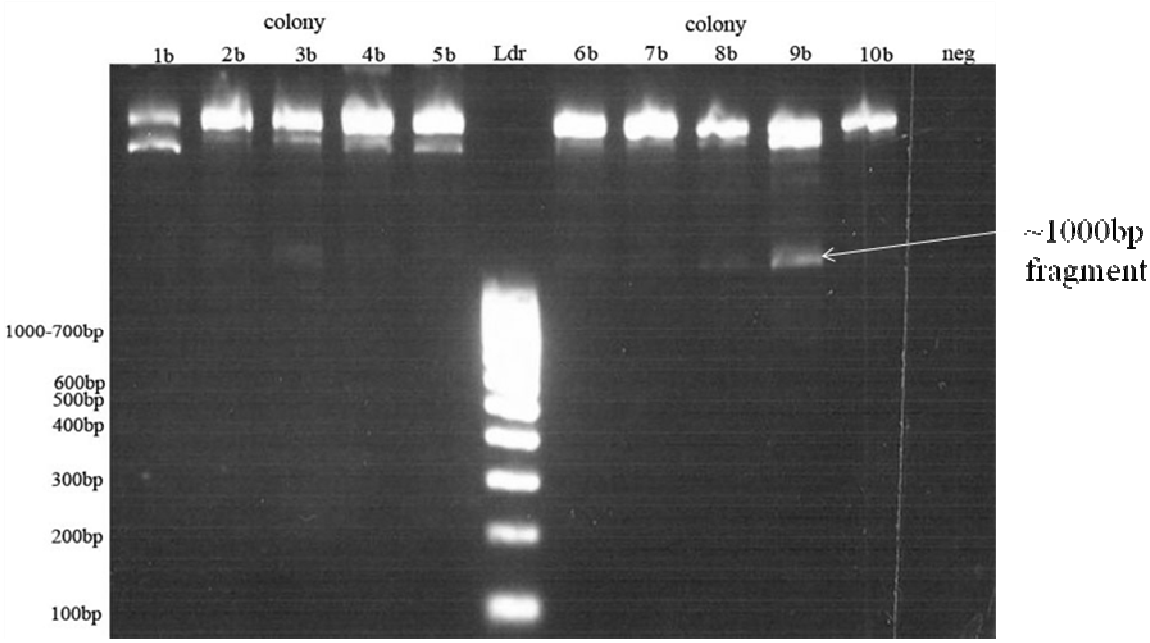
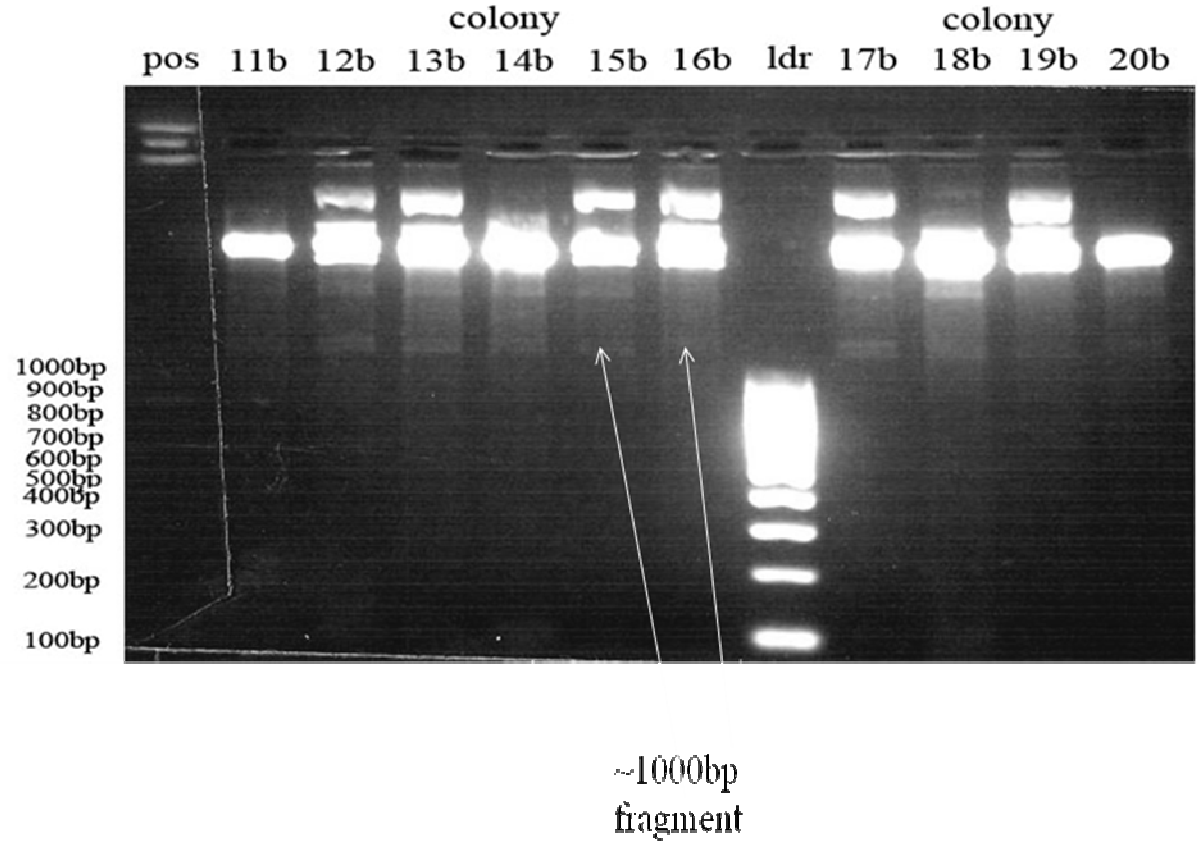


Figure 3.25 – Second TOPO transformation (second 10 colonies)

Showing a universal uptake of >1000bp fragment



The consistent failure of sequencing attempts indicates the DNA was of insufficient quality, or that it is non-coding 'junk' DNA which will not amplify with standard vector primers. Sequencing attempts on the PCR product using in-house primers also failed.

A very high number of positive (white) colonies was observed throughout the TOPO transformations, therefore to eliminate the possibility of false positives from the transformations, second ligations were re-transformed into TOP10 cells and plated on agar containing 50µg/ml kanamycin in addition to ampicillin, X-gal and IPTG and incubated overnight at 37°C. No colonies resulted from this incubation indicating that ligation was in fact unsuccessful, and that there may have been a problem with the batch of TOP10 cells.

Unfortunately due to the considerable time spent on this work, and technical constraints described here, it was decided that no further investigation should be conducted along this line.

3.4 Discussion

3.4.1 Streptococcal pyrogenic exotoxins A, C, L and M

3.4.1a SPEA

rSPEA was successfully expressed (Figure 3.1) and validated by the demonstration of Vβ specific T cell expansion following stimulation of PBMCs. It was decided that the optimal gating strategy to use was to gate on blasting and resting lymphocytes separately, as opposed to gating on all T-lymphocytes (Figure 3.2 and 3.3). This strategy was used in all further Vβ proliferation assays. Using this strategy it was observed that as concentration of SPEA decreased the percent of Vβ14 positive T cells as a proportion of the total CD4+ blasting lymphocyte population increased, suggesting that Vβ14 is the primary Vβ stimulated by SPEA. As concentration of SPEA increased, the proportion of Vβ14 decreased, which is due to the expansion of other Vβs (3, 12, 13) not studied here, and consistent with the findings of Llewelyn *et al* who described the spreading of TcR Vβ signature for several superantigens with increasing stimulus strength [73].

3.4.1b SPEC

A plasmid encoding GST-SPEC was transformed into BL21 (pLys) DE3 cells and gene expression induced using IPTG (Figure 3.4). Initially due to incorrectly labelled plasmid PreScission Protease was used to attempt to cleave GST from SPEC. This was unsuccessful, but subsequently trypsin was successfully used to cleave GST from SPEC (Figure 3.5) A Vβ proliferation assay was conducted on recombinant SPEC (Figure 3.6) which indicated a Vβ

specific expansion in Vβ8 and to a lesser extent Vβ12. No expansion was seen in Vβ2, contrary to documented evidence that SPEC expands Vβ2 [69]. This evidence was obtained using reverse transcription of Vβ genes following stimulation with recombinant SPEC, in contrast to the flow cytometric approach used in this research [69]. This variation in Vβ signature has been previously observed for SPEA by Llewelyn *et al* who demonstrated expansion in Vβs 3, 12, 13 and 14 in response to SPEA [73] in contrast to the previously recognized signature of Vβs 2, 12, 14 and 15 [7]. That this has been shown for one streptococcal superantigen indicates that the same may be the case for SPEC. The variation observed here may to a certain extent be caused by differences in methods used, for example some of the original Vβ signatures, including that for SPEA and SPEC were initially determined using PCR [69], whereas these results were obtained by flow cytometry. TcR Vβ regions can be grouped based on their genetic similarities (Figure 1.4 above) [79]. Based on current evidence SAgs expand Vβ regions which share more homology within their sequences, over Vβs which share less. There may be more subtle differences between TcR Vβ regions which cannot be defined by phylogeny which may also contribute to the differing Vβ signatures observed between researchers.

3.4.1b SPEL and SPEM

Plasmids encoding Trx fusion SPEL and SPEM were successfully transformed into competent BL21 (pLys) DE3 cells and expressed (Figure 3.4). Protein tags were linearised from SPEM on the first attempt and SPEL on the second attempt using PreScission Protease (Figure 3.5). Possible reasons for the initial unsuccessful removal of Trx from SPEL include enzyme degradation or sub-optimal digest conditions. A low yield of SPEL may also have accounted for this problem.

SPEL showed the predicted expansion of Vβs 1 and 23 and to a lesser extent Vβ5.1 [58] (Figure 3.7). Proportions of all Vβs studied decreased with concentration of stimulus, in contrast to SPEM, although at a lower range of concentrations of SPEL the proportion of Vβ1 and 5.1 increased again, suggesting a high level of toxicity at greater concentrations, accounting for the unusual results observed at higher concentrations (Figure 3.7).

SPEM showed a predicted expansion in Vβs1, 5.1, and 23 (Figures 3.8-10) [58]. Llewelyn *et al* have previously studied the relationship between superantigen stimulus strength and the specificity of Vβ specific T cell responses. They have demonstrated, for SEA, SEB and SPEA that as stimulus strength (concentration, HLA class II type) increased, the range of Vβ types responding to a SAg broadens. Conversely by looking at Vβ specific responses at very low SAg concentrations it is possible to determine which Vβ types are targeted by a superantigen at the very low concentration likely to be present at a site of infection [73]. Using this approach it was found that Vβ specific T cell responses to SPEM occur down to

0.1fg/ml and that these target Vβ1, suggesting that this may be the primary Vβ type responding to this superantigen for these donors. This can be interpreted as a genuine response as opposed to contamination, as even at this low concentration approximately 2200 molecules of toxin were present in wells (using Avogadro's constant of 6.022×10^{23} and the Mw of SPEM as 27.4kDa). A further interesting aspect of these data is that while the predicted responses of Vβ5.1 and 23 were seen, these varied markedly among the three individuals tested. Llewelyn *et al* demonstrated that differences in HLA class II resulted in approximately 1 log dilution differences in the relationship between superantigen concentration of T cell response, both in terms of magnitude and Vβ specificity [73] Different responses to superantigens attributed to HLA type have also been noted by Norrby-Teglund *et al* and Hermann *et al* [65, 67]. Data presented here could indicate the response to SPEM is much more heavily influenced by HLA class II than for other SAgs. This was investigated further and is described in Chapter 6 below. A further observation made here was that SPEL and M responses seemed to vary from day to day in the same donor (Figures 3.7-10c). It is unknown why this may be the case, although it could indicate that factors other than HLA type may contribute to SAg response. This phenomenon is described further in Chapter 6.

It is interesting to note that SPEL and SPEM used here target the same Vβs but with very different potencies (P_{50} SPEL is 1pg/ml, SPEM is 10pg/ml) [58]. This suggests in examples where both toxins are present in the same strain the two could work synergistically to enhance each other's effects and obtain the optimal response from the host, reinforcing the suggestion that HLA haplotype restrictions may have driven the evolution of multiple SAg genes [73].

3.4.2 Expression of staphylococcal enterotoxin J

The staphylococcal enterotoxin SEJ was chosen as a novel SAg for cloning as its successful expression had not been reported in the literature. As described above, considerable technical difficulties were faced in the process of this work. However repeated attempts to clone *sej* gene into two different vectors have failed, despite various modifications to the techniques used.

Optimisation of PCR proved relatively simple, although did require quite a high concentration of magnesium which may have increased the risk of non-specific amplification. Once optimised, PCR produced consistent results. Digest of vector and insert appeared to be successful and verified by agarose gel electrophoresis. However ligation into pPROEX-HTb was unsuccessful resulting in no colonies over numerous attempts. This may have indicated unsuccessful digestion of vector or insert, or insufficient quantity of DNA from gel excision and purification. Therefore a decision was made to use TOPO pCR 2.1

for sequencing and to subclone from here into pPROEX-HTb. Ligation of PCR product into TOPO pCR 2.1 was successful but resulted in a very high number of positive colonies. This may have indicated there was a problem with the batch of TOP10 cells used, for example they may have lost the antibiotic resistance gene. Alternatively there may have been mutational damage to or loss of the lac operon controlling the blue/white selection, resulting in an abnormally high proportion of positive colonies. Additionally, all attempts to sequence products from positive TOPO colonies failed, indicating insufficient DNA quality or quantity, which perhaps reinforces the possibility of false positives.

In addition to the possible technical problems with this work, it is interesting to note that although the gene for SEJ was identified several years ago [176], until very recently there was no published evidence of it ever having been cloned. Thomas *et al* recently used a pQE30 plasmid vector and expression in *E. coli* M15 cells to clone SEJ and other staphylococcal superantigens using the strain of *S. aureus* in which SEJ was originally identified to amplify the target gene (Fukuoka 5, [176, 177]). This group used a different strain of *S. aureus* as well as different vectors and *E. coli* for their cloning work to that described in this chapter, however it is not clear from the research if this group also encountered problems in the cloning of SEJ [177]. Generally upon the identification of a novel superantigen the gene is cloned and characterised immediately [7, 30, 74, 164, 178]. That this was not done immediately with SEJ suggests there may be a particular characteristic of *sej* which renders it difficult to clone, for example it may be that *sej* is palindromic and forms secondary structures when isolated [179]. It is also possible that *sej* is toxic to *E. coli,* and therefore is deleted by intracellular mechanisms [179]. Following considerable trouble shooting on this research, and no resolution of the problems described it was decided in the best interests of the project to move on from this work.

3.5 Conclusions

The work in this chapter allows several conclusions to be drawn:

- The optimal gating strategy to use for the assessment of Vβ profiles is to gate on blasting and resting lymphocytes separately.
- This shows a dosage dependent Vβ14 expansion with SPEA
- SPEC causes an expansion in Vβ12 and Vβ8
- SPEL and M demonstrate similar Vβ signatures (Vβs 1, 5.1 and 23) however responses differ between individuals and between the same individual at different times.
- Cloning of the novel staphylococcal superantigen SEJ was unsuccessful despite considerable trouble-shooting and modifications to technique.

4. Purification of HLA class II from HLA homozygous B cell lines

4.1 Introduction

Whilst superantigens are serologically diverse, that HLA class II remains their definitive receptor raises the possibility that the HLA class II structure could be exploited to develop novel superantigen ligands. The ultimate aim of this would be to develop novel structures or peptides demonstrating a wide specificity and high affinity for a range of SAgs.

Under normal circumstances SAgs bind their receptors, the HLA class II molecule and the T cell receptor, in a cellular environment. However crystallographic studies [32, 33, 45, 86] and surface plasmon resonance (discussed further in Chapter 5 below) [85, 180, 181] show us that binding can be demonstrated in a cell free environment. Leder *et al* performed an analysis of SEB and SEC3 mutated proteins and their binding to soluble HLA class II and TcR Vβ. This research demonstrated the differing affinities of superantigens to both receptor molecules, for example SEB binds HLA-DR with a K_d of 14μM, whilst SEC3 demonstrates a K_d of 48μM, despite the two superantigens sharing very similar three dimensional structures. The mutational analysis of this work also highlighted the importance of small sequence differences between SAgs as mutant proteins had far weaker binding affinities for both the HLA class II and TcR Vβ region. [181]. This work indicated the importance of broad specificity when developing novel superantigen ligands.

Using a slightly different approach and the so called B cell 'superantigens', Enever *et al* described a phage display technique for the development of high affinity superantigens. Bacterial cell wall proteins isolated from *Peptostreptococcus magnus* (Protein L, PpL), *S. aureus* (Protein A, PrA) and groups C and G streptococcus (Protein G, PrG) demonstrate a high affinity for the variable κ light chain or Fc regions of human immunoglobulin respectively. These proteins have been termed B cell superantigens for this reason and that they cause similar effects in B cells to the conventional superantigens actions on T cells. [126, 127, 129]. Enever *et al* developed a set of PpL ligands which had high affinity and broad specificity for human immunoglobulin [129], indicating that the same approach could potentially be used to develop the HLA class II molecule as a capture molecule for classical 'T cell' superantigens.

The work described in this and the following chapter aimed to generate proof of principle that soluble HLA class II can be used in this way. However, generation of data establishing the characteristics of superantigen binding by HLA class II molecules in a cell free environment – sensitivity and specificity for a range of superantigens exemplifying different

modes of interaction with HLA class II – requires milligram quantities of a range of different HLA class II molecules. Therefore the primary aim of this chapter was to generate the range of HLA class II molecules required.

Previously several approaches have been used to obtain HLA class II in solution, including affinity purification of HLA class II from HLA homozygous B cell lines and expression of complete or incomplete HLA class II. Commercially available soluble HLA-DR monomers and tetramers also exist. The advantages and disadvantages of each method are described below and summarised in Table 4.1 below.

Affinity purification of HLA class II is probably the oldest technique which exists. First devised in 1987 by Gorga *et al*, purification involves generation of a series of affinity columns utilising monoclonal antibodies against HLA class I (W6/32), HLA-DR (L243), and if desired, HLA-DQ, DP and DM. HLA is purified from detergent solubilisations of HLA homozygous B cell membranes. [182]. This technique has the advantage of producing a high yield (10s of mgs) if successful. The diversity of HLA homozygous B cell lines which are commercially available through the European Collection of Cell Cultures (ECACC) also means a wide variety of HLA serotypes can be purified using this method. HLA class II purified in this way can be stored at -20°C and can be thawed multiple times with no detectable effect on function. HLA class II purified using this method is peptide loaded, however for superantigen research this is of no great importance as SAgs bind outside the peptide binding groove [8]. This is however a technically challenging and prolonged technique involving purification of milligram quantities of monoclonal antibody, either via tissue culture of hybridoma cell lines or ammonium sulphate precipitation from ascites fluid, generation of columns and solubilisation of B cell membranes [182].

Various methods to express recombinant HLA class II are described in the literature, utilising eukaryotic cell lines (yeast *Pichia pastoris* [183]), prokaryotic cells (*E. coli* [184]) and insect cell lines (Ni Moth, *Trichoplusia ni* [185], *Drosophila melanogaster* [186]). Conventionally, as the HLA class II subunits are quite large genes, traditional bacterial plasmids cannot usually be used. Therefore cosmids are used in yeast systems [183] and baculoviruses in other approaches [185, 186].The sequences of HLA class II are widely available [39], meaning identification and amplification of HLA class II can be relatively simply achieved, allowing the generation of a wide range of recombinant plasmids. Once set up again this technique can provide a high yield and diverse range of HLA haplotypes, and additionally allows mutagenesis where needed. However as HLA class II is a transmembrane protein in its native state achieving correct folding *in vitro* can be very challenging. Most recombinant HLA class II is expressed as separate subunits and heterodimerised in solution [183-185], although single chain expression has been achieved

[187]. Researchers have found that HLA class II will only heterodimerise in solution if the transmembrane domain is replaced with a leucine zipper domain [183], further complicating the cloning process. Additionally, Bonnerot *et al* described the possible aggregation of soluble HLA class II when invariant chain (Ii) is not present, which could contribute to problems with this technique [188]. Finally, this technique requires specific tissue culture facilities, particularly if yeast or insect cultures are used, which may be expensive to set up and difficult to access. The potential exists to express recombinant partial HLA class II using this method, however folding problems would remain, as would problems with specific tissue culture facilities.

Commercially available HLA class II has the obvious advantage that it removes technical difficulties associated with other techniques. However HLA class II has only become commercially available in recent years, probably due to the difficulties in producing large quantities. Currently only a handful of companies manufacture HLA class II (ProImmune UK, Beckman Coulter UK, NIH Tetramer Facility, Atlanta GA) and it remains incredibly expensive. Purchasing milligram quantities of HLA class II is virtually impossible as most companies only supply microgram quantities. The range of serotypes available is also very limited, making it challenging to assess the best HLA class II serotype in terms of broad specificity and high affinity for SAgs.

Table 4.1 Merits of various methods of obtaining soluble HLA-DR

Data broadly derived from refs [182-188]as in above paragraphs

Affinity purification of HLA-DR		**Commercially available HLA-DR**		**Recombinant complete or incomplete HLA Class II**	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**	**Advantages**	**Disadvantages**
1. Wide range of DR serotypes available from B cell lines 2. Also possibility of purifying class I for use in other assays 3. Possibility of purifying DQ with an additional column 4. High yield if successful 5. Cost effective 6. Can be stored at -20	1. Technically challenging 2. Takes one month from cells to DR if successful 3. Cost ineffective to set up 4. Labour intensive technique	1. Quick 2. No technical challenges	1. Very limited range of serotypes 2. Usually only available as tetramers 3. Only available in very small amounts 4. Very cost ineffective 5. Six week lag time between ordering and receiving 6. Stored at -80 if monomers	1. Wider range of serotypes than commercial 2. Could potentially just express α or β chain, or short peptides 3. Easy of purification 4. Potentially high yield 5. Relatively cost effective	1. Use of specific insect cell culture techniques 2. Initial set-up may be challenging 3. Possible problems with correct folding of molecule

Bearing all the above in mind, it was decided that the optimum method to use for the purification of milligram quantities of HLA class II in order to obtain the basic proof of principle described above was affinity purification. The following will describe the methods used and results of this technique.

4.2 Methods

Buffers used were as described in Table 2.1 above unless otherwise specified in particular methods. All buffers used in columns were degassed for 30 minutes and filtered through a 0.45µM filter before use.

4.2.1 Purification of Antibodies from Hybridoma Cell Lines

Binding Buffer: 20mM sodium phosphate pH7 – (GE healthcare)

This was made from a combination of sodium phosphate buffers according to manufacturers' instructions as 57.7% monobasic sodium phosphate (buffer A) to 43.3% dibasic sodium phosphate (buffer B).

- Buffer A: 1M Na_2HPO_4
- Buffer B: 1M NaH_2PO_4
- 1M sodium phosphate buffer pH7: 57.7% buffer A: 43.3% buffer B.
- Binding buffer: 20mM sodium phosphate from 1M stock.

Elution Buffer: 100mM sodium citrate pH4

This was made using 85ml 0.2M sodium citrate, pH adjusted with 165ml 0.2M citric acid, and made up to 500ml using dH_2O to give a 0.1M solution **[189]**.

- Buffer A: 0.2M sodium citrate:
- Buffer B: 0.2M citric acid
- Elution buffer: for 500ml: 85ml 0.2M sodium citrate added to 165ml 0.2M citric acid, made to 500ml using dH_2O.

Neutralisation Buffer: 100mM Tris-HCl pH9

5ml HiTrap rProtein A Fast Flow columns (GE Healthcare, UK) were used to purify monoclonal antibodies from the supernatant of HB95 and HB55 cells as follows:

1. HB95 and HB55 cells were maintained and harvested as described in Chapter 2.

2. Supernatants were diluted 4 parts supernatant to 1 part binding buffer.

Antibodies were purified from supernatant as follows:

1. Storage buffer was removed using 30ml binding buffer
2. Columns were connected drop-drop to the peristaltic pump.
3. Columns were regenerated using elution buffer at <5ml/minute
4. Antibody samples were applied at 5ml/minute
5. Sample was washed through using binding buffer
6. Antibody was eluted in 20 fractions using elution buffer. Each fraction was neutralised with 150µl neutralisation buffer
7. Columns were washed using binding buffer and stored in 20% ethanol at 4°C
8. Absorbance 280 and 260 of fractions was measured and protein concentrations estimated using either F_1 or F_2 below.

F_1: $C = A/\varepsilon$ (mg/ml) where IgG is 160000 and ε is 14.3. This formula applies if A_{280}/A_{260} is < 0.6

OR

F_2: $C(mg/ml) = (A_{280} \times 1.55) - (A_{260} \times 0.77)$

Fractions containing >0.2mg/ml protein were pooled and dialysed overnight against 1 x PBS 0.05% sodium azide. Following dialysis accurate protein concentration was calculated using BCA and purity assessed using SDS-PAGE. Samples were concentrated in Centricon centrifugal devices and stored at ~1mg/ml at 4°C.

4.2.2 HLA-DR Enzyme Linked Immunosorbent Assay (ELISA)

Buffers

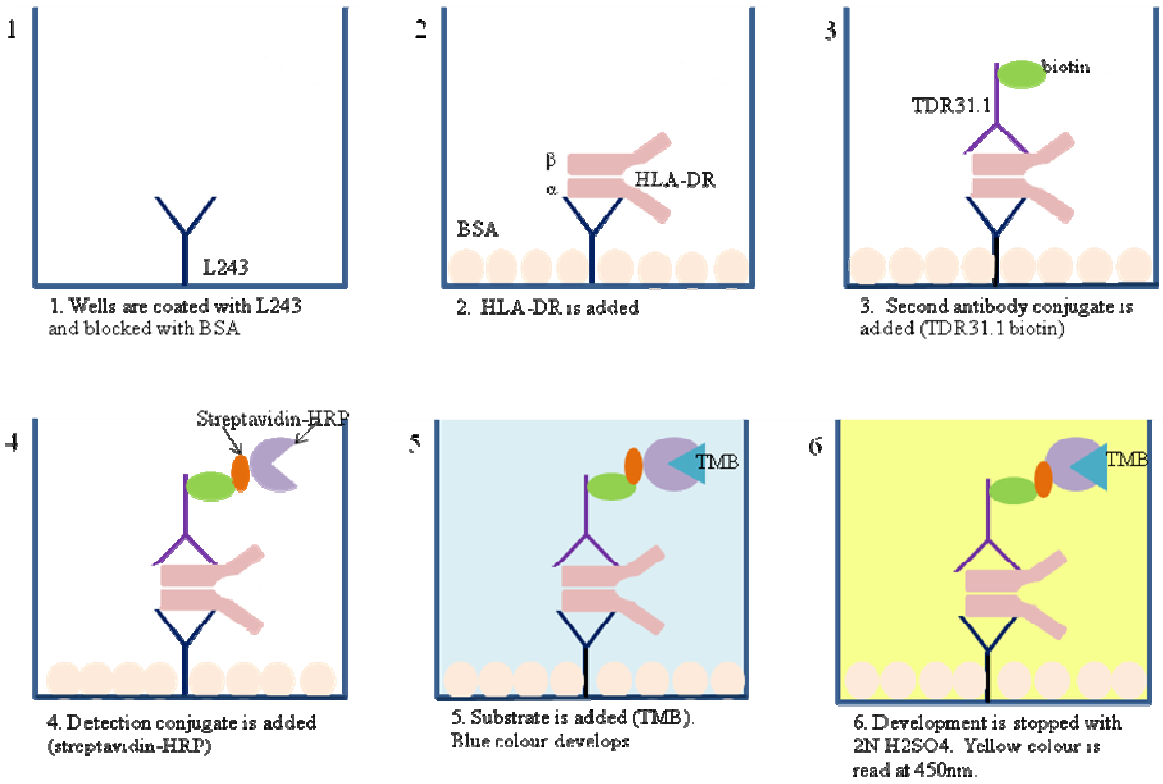
HLA-DR standards: 2µg/ml HLA-DR15 prepared in blocking solution. Starting concentration 2µg/ml, 8-12 1:2 serial dilutions of this in duplicate.

As a tool to allow quantification of purified HLA-DR An ELISA for the detection of HLA-DR in solution was developed using purified HLA-DR15 as a standard and antibodies specific for HLA class II α and β chain. Figure 4.1 below shows the principles of this assay.

Procedure:

1. 96 well flat bottomed plates (Corning, UK) were coated overnight at 4°C with 50µl/well 1.6µg/ml L243 antibody in carbonate buffer pH 9.0 (panel 1, Figure 4.1).
2. Plates were washed 5 times using 200µl/well wash buffer.
3. Wells were blocked overnight at 4°C using 300µl blocking solution/well.
4. Plates were washed. Duplicates of 50µl HLA-DR standards were added alongside samples and blanks (panel 2)
5. Plates were incubated for 1 hour at RT and washed.
6. 50µl/well 1µg/ml second antibody conjugate (TDR31.1-biotin) was added and incubated for 1 hour at RT (panel 3).
7. Plates were washed and 50µl/well 0.5µg/ml streptavidin-horseradish peroxidase (HRP) added. Plates were incubated for 1 hour at RT (panel 4) and washed.
8. 100µl/well tetramethyl benzidine (TMB) substrate was added (panel 5)
9. Reactions were stopped after 15 minutes using 100µl/well 2N H_2SO_4
10. Yellow colour was read at 450nm (panel 6) in a Synergy plate reader using KC4 software to analyse and results plotted.

Figure 4.1 ELISA for the detection and quantification of soluble HLA-DR in samples



4.2.3 Coupling monoclonal antibody to Sepharose Columns

W6/32 and L243 were conjugated to both pre-packed NHS-activated 5ml HiTrap Sepharose columns (GE Healthcare), and protein A Sepharose which was packed into columns following conjugation. Generation of both types of columns utilised similar methods, which are described below.

4.2.3a Coupling monoclonal antibody to NHS activated 5ml HiTrap Columns

Buffers

Buffer A: 0.5M ethanolamine, 0.5M NaCl pH8.3

Buffer B: 0.1M acetate, 0.5M NaCl pH4

Ice cold 1mM HCl

Conjugation buffer: 0.2M $NaHCO_3$ 0.5M NaCl pH 8.0

Coupling was carried out according to manufacturer's instructions as follows:

Antibody at 5-10mg/ml was dialysed overnight at 4°C into conjugation buffer. Ice cold HCl was then applied to columns and storage isopropanol washed out drop by drop. 5ml antibody solution was injected into columns and columns sealed for 45 minutes at RT. Following conjugation excess active groups were deactivated and excess ligand removed using the following protocol:

a. Inject 3 x 10ml Buffer A
b. Inject 3 x 10ml Buffer B
c. Inject 3 x 10ml Buffer A
d. Let the column stand for one hour
e. Inject 3 x 10ml Buffer B
f. Inject 3 x 10ml Buffer A
g. Inject 3 x 10ml Buffer B
h. Inject 10ml 1 x PBS 0.01% azide

These columns were connected in series with a non specific 5ml Protein A HiTrap column (GE Healthcare).

4.2.3b Coupling monoclonal antibodies to Protein A Sepharose

Buffers

Borate Buffer: 100mM boric acid pH 8.2.

Triethanolamine buffer: 200mM triethanolamine pH 8.2

Dimethyl pimelimidate (DMP) cross-linking buffer: 200mM triethanolamine containing 20mM DMP.

Ethanolamine Buffer: 20mM ethanolamine. 1.2ml ethanolamine diluted in 1L dH_2O.

Procedure

Columns of 5ml bed volume were prepared by coupling 20mg monoclonal antibody to Protein A sepharose (Zymed, Invitrogen, Carlsbad CA). 5ml pre-swollen Protein A sepharose was suspended in an equal volume of borate buffer, and washed 4 times by centrifugation. Protein A sepharose was resuspended in borate buffer as a 50% slurry. Antibody was diluted to 50ml in borate buffer and coupled to Protein A sepharose overnight at 4°C with rocking. Antibody coupled resin was poured into Kontes columns (Sigma-Aldrich, Waltham, MO) and settled under gravity. Columns were washed with 2 column volumes (CVs) borate buffer, and the process repeated until no more protein was eluted (determined by A_{280}).

Columns were washed with 2 x 25ml triethanolamine buffer and resuspended in 20 x CV of the covalent cross-linker DMP buffer for 45 minutes at RT. Antibody coupled resin was poured into the columns and settled under gravity at 4°C. Columns were washed using 25ml ethanolamine buffer and 50ml borate buffer.

A 10ml pre-clear column (sepharose-CL-4B, Sigma-Aldrich) and a 5ml non-specific protein A sepharose column were poured and washed with borate buffer.

In both cases, columns were stored at 4ºC in their relevant storage buffer, and washed with TrisDOC buffer prior to use.

4.2.4 Purification of HLA-DR from homozygous B cell lines

B cell lines were cultured as described in general methods (Chapter 2).

Buffers

Nonidet-NP40 (Igepal, Sigma-Aldrich St. Louis, MO)

Cell lysis buffer: 10mM Tris, 0.1mM PMSF, 1mM DTT pH8.0

MOPS NaCl buffer: 20mM MOPS 140mM NaCl 0.1% DOC pH 7.6

TrisDOC buffer: 10mM Tris 0.1% DOC pH8

Glycine buffer (*elution buffer*): 50mM glycine 0.1% DOC pH11.5

Glycine Neutraliser Buffer: 2M glycine pH2

Tris-NaN_3-Igepal buffer (storage buffer): 10mM Tris 0.1% Igepal 0.05% NaN_3 pH7.8

Procedure

HLA-DR homozygous B-cell pellets were thawed in a 37°C water bath until they could be removed from tubes into a glass flask on ice. Tubes were washed using ice cold cell lysis buffer and thawing continued in this buffer. Cell suspension was homogenised using 5 strokes of the mortar to 30mls. Homogenised cells were incubated for 30 minutes at 4°C with rocking.

The suspension was centrifuged for 5 minutes at 4000 x g. Pellets were yellowish and loose, and supernatant cloudy. Supernatant was decanted and stored on ice whilst the pellet was resuspended in 20ml cell lysis buffer and re-spun up to 8 times until the pellet was white and the supernatant translucent. Combined supernatants were centrifuged (175000 x g, 40 minutes, 4°C).

Pellets were homogenised and membrane proteins extracted by addition of NP40 dropwise over 30 minutes, and rocking for 30 minutes. The suspension was then centrifuged at 175000 x g for 2 hours at 4°C in a pre-cooled rotor. Supernatants were pooled and applied to four affinity purification columns; non-specific sepharose CL-4B, protein A sepharose, anti-HLA class I (W6/32) and anti-HLA DR (L243). Columns were pre-cleared using 2 CVs MOPS buffer, 5 CVs TrisDOC, 2 CVs glycine buffer, 2 CVs TrisDOC and 2 CVs Tris-Igepal-NaN_3.

B cell membrane extract was applied to the column at 3ml/hour. Extract was followed with 4 CVs Tris-Igepal-NaN_3, 2 CVs MOPS buffer and 4 CVs TrisDOC before elution with 10ml glycine buffer. Eluates were neutralised with glycine neutralisation buffer and dialysed against TrisDOC overnight before concentrating using Centricon-YM30 centrifugal devices. Samples were analysed using SDS-PAGE, BCA Protein Assays and HLA-DR ELISAs.

4.3 Results

The aim of this work was to purify conformationally–intact peptide–loaded HLA-class II molecules by affinity column purification from membrane preparations of B cell lysates (described above), in addition to developing a technique for the validation of purification.

4.3.1 Optimisation of HLA-DR ELISA

Methods above describe the ELISA used for quantification of soluble HLA-DR in column eluents, however this method was optimised over the course of several months using existing stocks of HLA-DR15. This will be described below.

As an initial proof of principle high concentrations of L243 were used to coat the plates to obtain a working standard curve of detectable HLA-DR. Initial investigations demonstrated inconsistency between duplicates and between experiments. To improve this various washing procedures and pipettes were used. Very little difference was observed between washing plates using a multichannel pipette or a 'buffer bath', however consistency was greatly improved by incubating plates in wash buffer for 1 minute in between washes. Therefore plates were washed 5 times with a minute incubation in between each wash, using a multichannel pipette in all subsequent experiments. The consistency between duplicates and repeatability between experiments was also improved by the use of a multichannel pipette over a multistep pipette.

In initial investigations streptavidin-alkaline phosphatase (AP) and ρ-Nitrophenyl phosphate (PNPP, both from Pierce Biotechnology, Rockford IL) were used as a detection reagent and substrate, however following several experiments this was found to be inefficient and inconsistent, therefore these were replaced with streptavidin-HRP (Serotec, UK) and TMB (Thermo-Fisher Scientific). Following further investigations, performance of these reagents was further enhanced by stopping the reaction using 2N H_2SO_4 after 15 minutes incubation at room temperature. Plates were subsequently read at 450nm instead of 370nm.

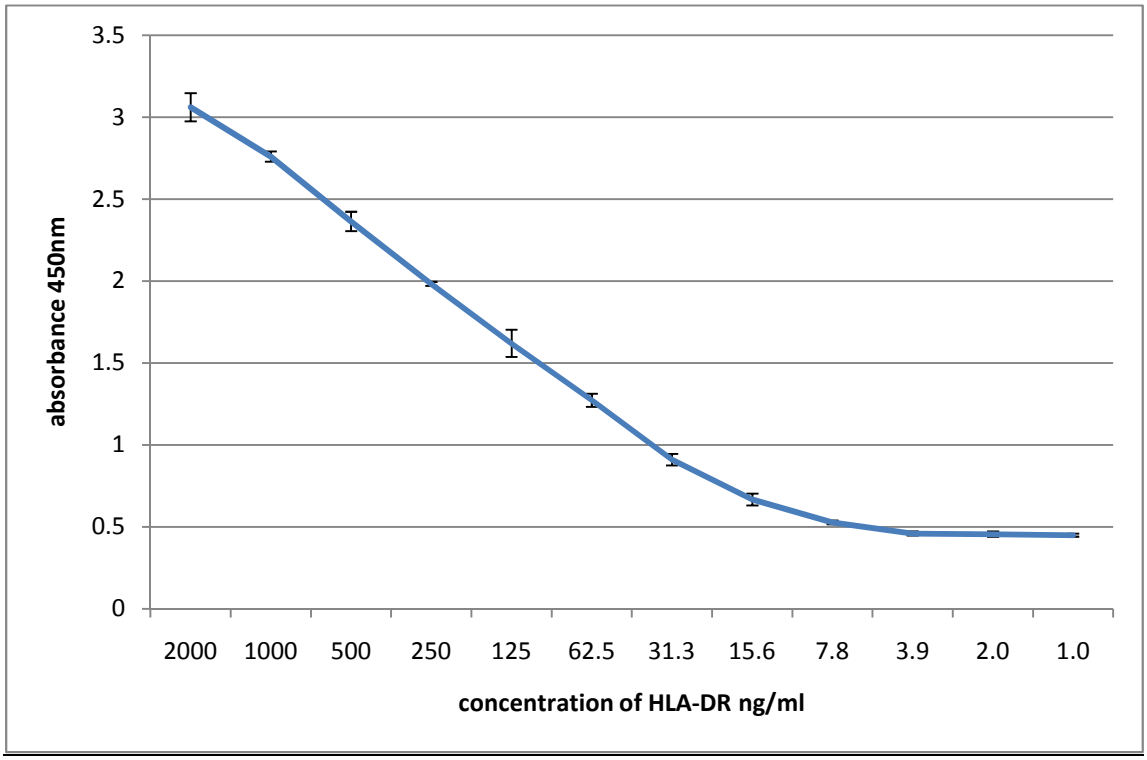
Once a repeatable standard curve of DR was achieved, L243 concentration was reduced over several experiments to obtain an optimal concentration of 1.6µg/ml. Investigations were also carried out simultaneously to determine the optimal coating buffer between 1 x PBS at pH 8 and carbonate buffer at pH 9. The most consistent standard curve was obtained using carbonate buffer, therefore this was used as a coating buffer for all subsequent experiments.

Following all these optimisations, the concentrations of HLA-DR the assay was repeatedly and consistently able to detect was within a range of 2000ng/ml to 15ng/ml. This is demonstrated in Figure 4.2 below. Once optimised this assay was further developed for use in other applications. For example, standard curves were established using human serum inoculated with HLA-DR. This was then used to quantify soluble HLA-DR in sepsis patient serum

samples as part of a separate study. In the context of this study, this optimised ELISA was used to detect soluble HLA-DR in column eluents.

Figure 4.2 A typical standard curve from optimised HLA-DR ELISA

Showing a detectable range of HLA-DR from 2000ng/ml to 15ng/ml using TMB as a substrate. Points shown are a mean of duplicate readings +/- 1 standard deviation.



4.3.2 Purification of HLA-DR from HLA homozygous B cell lines

Following optimisation of the ELISA, work was undertaken to purify HLA class II from HLA homozygous B cell lines.

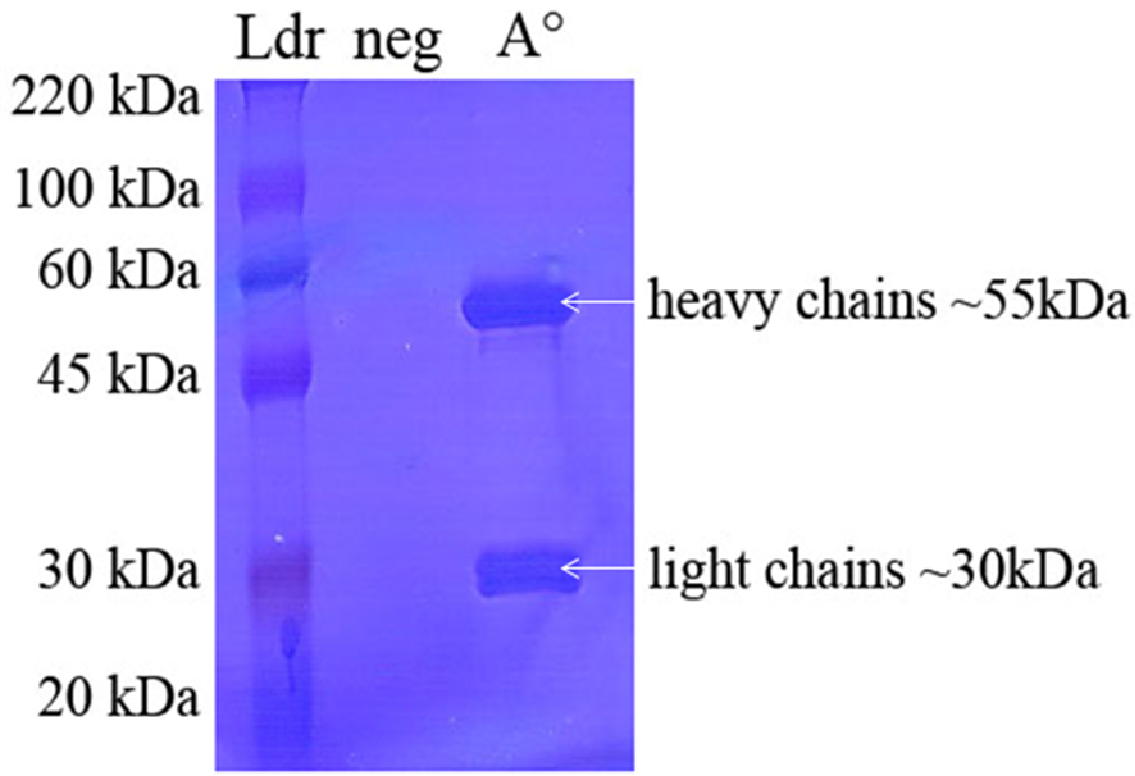
4.3.2a Antibody purification and NHS activated columns

HB95 and HB55 hybridoma cell lines were cultured as described in Chapter 2 and monoclonal L243 and W6/32 antibodies respectively purified from supernatants on protein A columns. Supernatants were initially passed over the columns multiple times, however this resulted in a negligible increase in yield, therefore further supernatants were only purified once. An initial purification yielded ~40mg L243, however only ~10mg W6/32 was purified on the first attempt so this was repeated to yield a further ~20mg antibody. Figure 4.3 shows a typical gel from the purification of monoclonal antibody.

Following purification of monoclonal antibody, L243 and W6/32 were conjugated to NHS activated Sepharose in pre-packed columns using the above method.

Figure 4.3 Dialysed monoclonal antibody (L243)

12% polyarylamide gel of purified monoclonal antibody showing a band of heavy chains at around 55kDa and a band of light chains at around 30kDa. Bands were visualised using coomassie blue stain.

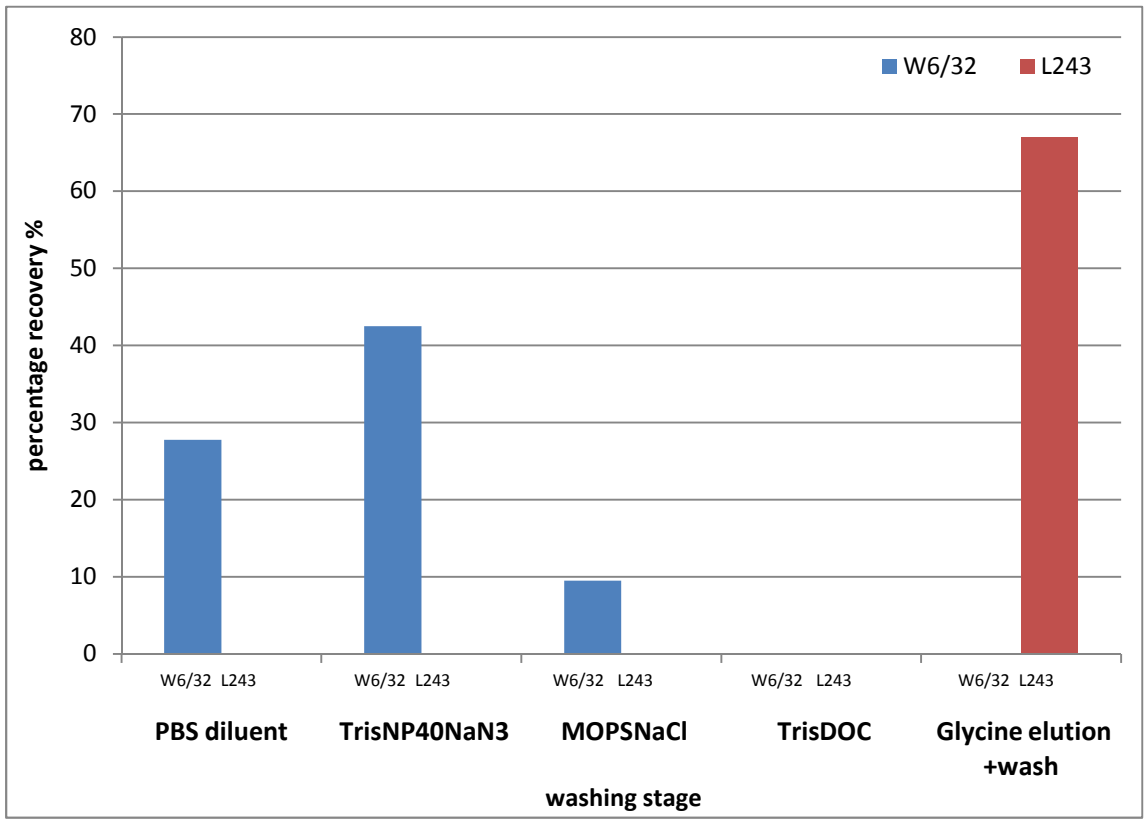


4.3.2b Validation of NHS activated columns and HLA-DR purification

To validate efficiency of HLA-DR binding to antibody conjugated NHS activated HiTrap columns a small scale purification was performed. A small quantity (100µg) of HLA-DR15 was applied to columns and followed by the washing and elution stages described above. Concentrations of HLA-DR present in washing and elution stages were determined using an HLA-DR ELISA as described. Results indicated a high level of efficiency (67% recovery of HLA-DR from L243 column, Figure 4.4). There was little non-specific binding of HLA-DR to the W6/32 column (79.7% recovery). HLA-DR was only eluted from the L243 column in the elution stage, indicating a specific binding of HLA-DR to L243.

Figure 4.4 Recovery of HLA-DR from W6/32 and L243 conjugated columns at various washing and elution stages

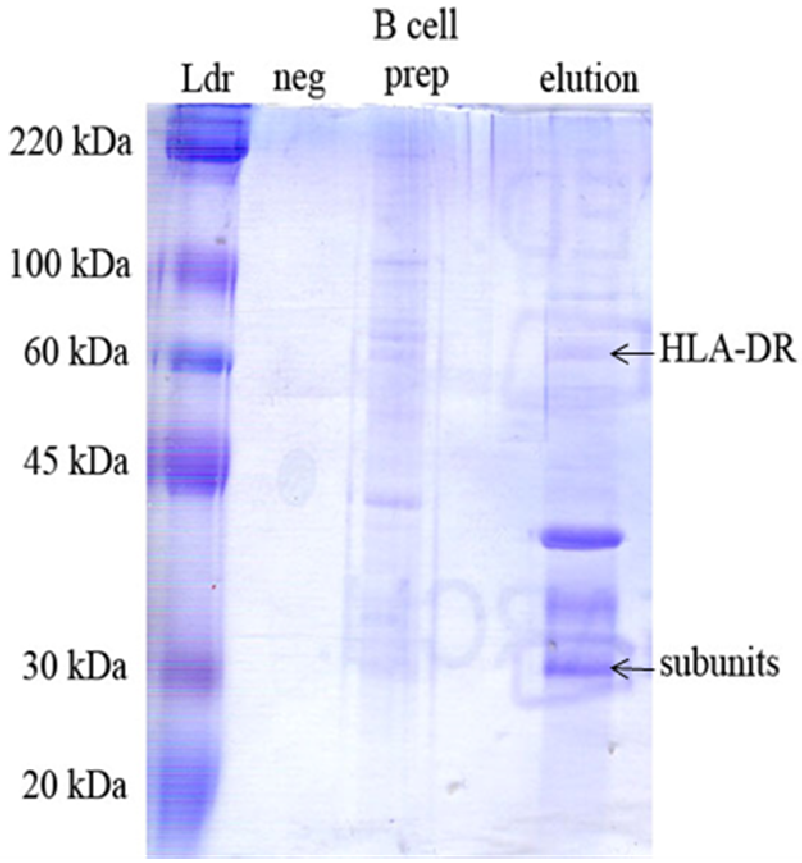
Results shown are percentages of HLA-DR recovery from a batch scale affinity purification of PGF B cell membrane lysate. 67% of HLA-DR applied was recovered in the elution stage of L243 column. No HLA-DR was eluted from this column in any other wash. In contrast 79.7% HLA-DR was recovered from the W6/32 column in preclearing and washing stages.



Following this successful validation a large scale purification was carried out on a B cell membrane preparation of PGF cells (HLA-DR15). The columns used in this purification also included a 10ml non-specific pre-clear column to remove irrelevant cellular proteins by gel filtration. The large scale purification initially revealed no detectable HLA-DR in either SDS-PAGE or the above HLA-DR ELISA. Therefore to eliminate the possibility that elution had not been efficient, all columns were eluted a second time using freshly made elution buffer. The pre-clear columns were also washed with an excess of binding buffer (up to 20 column volumes) to ensure no protein remained. This eluent was run on the L243 column again, and this column eluted for a third time. ELISA and protein assay analysis of all eluents and further washes revealed a very small quantity of HLA-DR in L243 eluents (~300µg) which was immediately concentrated in 30 MWCO Centricon Centrifugal devices but subsequent SDS-PAGE analysis of concentrated eluent indicated a very impure product (Figure 4.5). It was also apparent that during the concentration process some protein had precipitated out of solution. The fraction was filtered through a 0.45µM syringe filter, however subsequent analysis by SDS-PAGE showed no detectable HLA-DR.

Figure 4.5 SDS-PAGE of eluted HLA-DR

12% polyacrylamide gel showing eluent from an affinity purification of PGF B cell membranes. A single band of around 60kDa corresponding to HLA-DR and two bands around the 30kDa marker corresponding to the HLA-DR alpha and beta subunits are observed. The band at ~40kDa and other fainter bands are likely to be cellular protein contamination, indicating the purification was not optimal. Bands were visualised using coomassie blue stain.



Following this unsuccessful purification, a further validation identical to that described above was conducted on both the W6/32 and L243 columns using stock HLA-DR15. HLA-DR was present in all washes from columns in this assay, but not more so in elutions than in washes. This indicated the columns were no longer functioning correctly, and that either antibody had washed off the columns, or had been denatured by the purification process. Therefore hybridoma cell lines were cultured in large scale again in order to purify more monoclonal L243 and W6/32 to generate a second set of columns. PGF cells were also cultured again and frozen as cell pellets for a repeat purification.

In addition to confirm the stability of monoclonal antibodies in all chemical conditions required for purification a precipitation test was conducted of each antibody in each buffer, which failed to show any structural evidence of antibody degradation. Manufacturers of columns and hybridoma cell lines were also contacted and could offer no further advice.

4.3.2c Modifications to the preparation of HLA class II affinity columns

Before preparing a second set of columns time was spent observing affinity column generation and purification in the laboratory of Professor Mark Peakman with Dr Richard Ellis (Kings College London). Following this the technique used was modified in the following ways:

- Protein A sepharose (Zymed, Invitrogen, Carlsbad CA) was used in place of HiTrap columns
- To further stabilise binding of antibody to protein A sepharose a covalent cross-linking reagent (DMP) was used.

Following the purification of further monoclonal antibody from the supernatant of hybridoma cell lines, a batch test was performed on an aliquot of Protein A sepharose (Figures 4.6a and b). This involved binding a small amount of antibody to resin and washing with borate buffer and 1M NaCl to remove non-specifically bound protein. Antibody was then eluted from the resin using 0.1M glycine pH 2.7 and fractions run on a gel. Excess antibody was present in decreasing concentrations in borate buffer washes and NaCl washes, and present in elution fractions. A sample of resin was also loaded onto the gel after the batch test, and indicated some residual antibody still bound. This test showed that antibody bound the Protein A sepharose successfully, and could be eluted.

Larger columns were then generated using the same method and antibody permanently coupled to the resin with the use of DMP (Figure 4.7). SDS-PAGE analysis of these columns indicated the presence of antibody in washes prior to the DMP cross-linking, and not afterwards,

suggesting the successful coupling of antibody to the sepharose. Results shown are for W6/32 and representative of those for L243.

Figure 4.6 Batch test of W6/32 antibody and Protein A sepharose

12% polyacrylamide gels showing the results of a batch test of W6/32 antibody binding to and eluting from protein A sepharose. Protein bands were visualised using coomassie blue stain.

a) Showing excess antibody present in borate buffer washes in decreasing concentrations. Also showing no antibody in the non-specific NaCl wash.

b) Showing a small amount of antibody in further NaCl washes and antibody present in elution fractions 1-5. Also showing residual antibody bound to resin following elution.

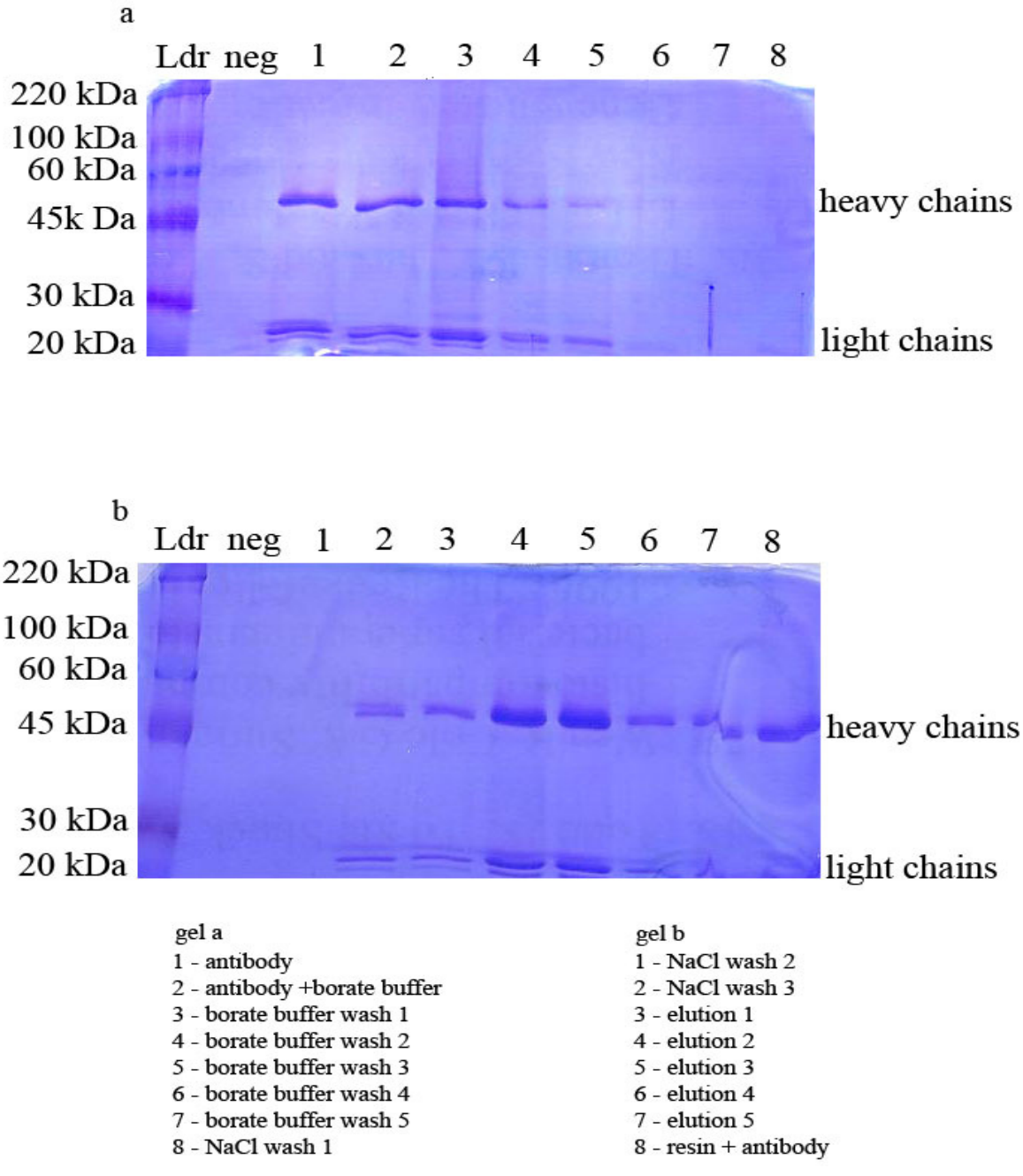
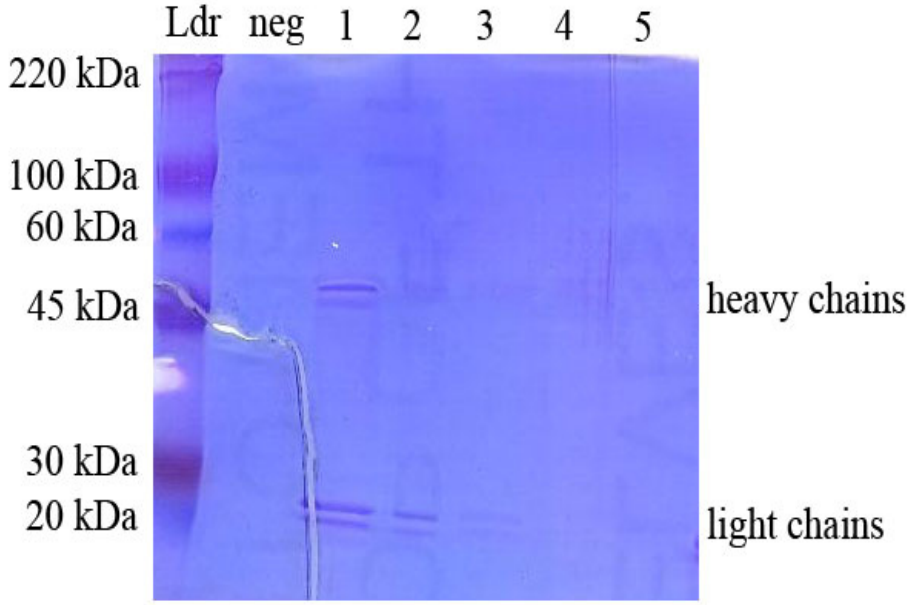


Figure 4.7 Formation of W6/32 column

12% polyacrylamide gel of column eluents following covalent crosslinking of monoclonal antibody to protein A sepharose. Showing antibody present in washes before but not after DMP cross-linking (Lane 3). Bands were visualised using coomassie blue stain.



1 - antibody
2 - 200mM triethanolamine wash
3 - DMP flow through
4 - 20mM ethanolamine wash
5 - borate buffer wash

Following successful regeneration of the columns, along with a 10ml non-specific sepharose CL-4B pre-clear column and a 5ml non-specific protein A pre-clear column to remove excess immunoglobulin from cell membrane preparations, a validation was conducted on antibody conjugated columns as described above. This was inconclusive, revealing no detectable HLA-DR in column eluents by ELISA or SDS-PAGE, although some washes appeared to contain antibody, indicating either excess unbound antibody, or that the cross-linking was not in fact successful. However, as stocks of HLA-DR15 were limited, validation was not repeated, and a large scale purification of PGF B cell membranes was carried out as above.

All column washes and elutions from all columns were subsequently subjected to SDS-PAGE analysis. This revealed no detectable HLA-DR in any wash or elution, and confirmed the presence of antibody in the original validation.

At this stage, due to the considerable technical constraints described, along with time and cost constraints it was decided to investigate alternative methods of purifying large quantities of HLA-DR.

4.4 Discussion

The work described above aimed to purify soluble HLA class II from HLA homozygous B cell lines using affinity columns generated in-house. Despite repeated attempts to purify HLA class II and modifications to the approach, purification of HLA class II in usable quantities was unsuccessful. The small quantity successfully purified was very impure and precipitated quickly out of solution, suggesting denaturing conditions or instability of protein. This is a technically challenging technique and there are several potential reasons why it may have failed.

There is a possibility that the monoclonal antibody used in affinity columns was degraded. This could be due to the fairly extreme pH conditions used during purification, although the precipitation test carried out indicated that this should not be the case. All antibodies used withstood extreme pHs used with no sign of precipitation. High pressure was applied to the columns throughout the purification process. Pressure has been documented to cause protein denaturation, so this must be considered as a possibility, [190] although such affinity columns have been used in previous experiments [66, 182, 191], and have been used multiple times with no reported loss in efficiency.

Column elutions from the second set of columns indicated the presence of antibody. This could either have been excess unconjugated antibody, or conjugated antibody which had been removed from the columns by elution. A sample of resin applied to an SDS-PAGE gel indicated that antibody was still conjugated to the resin, but this may have been a lesser concentration than originally cross-linked. A further possibility is that the cross-linking was

initially inefficient, resulting in most of the antibody applied being washed off the column immediately afterwards, although washes immediately after cross-linking suggest this is unlikely.

A further possibility exists that HLA class II became non-specifically bound to pre-clear columns. Additional washing of both pre-clear columns should have removed this possibility; however the additional washing may also have diluted any HLA-DR to undetectable concentrations. Subsequent purifications on pre-clear washes revealed no detectable HLA-DR, indicating either those additional washes were not sufficient to remove bound HLA-DR, or HLA-DR was not bound to pre-clear columns.

During the detergent solubilisation of membrane proteins, a protease inhibitor PMSF was used in buffers. A further possibility is that this protease inhibitor was denatured or functioning sub-optimally, resulting in the presence of cellular proteases in the B cell lysates. This may have resulted in the denaturation and digestion of solubilised HLA class II. This possibility cannot be ruled out, although again this problem has not previously been reported with this technique [66, 182, 191]. A final potential problem may have been that HLA-DR was not solubilised in detergent extracts. Western Blot analysis could be performed on B cell lysates to determine if this was the case, however as the detergent extraction is a long process involving several hours incubation, along with several hours ultracentrifugation, and the extract must be applied immediately to the columns, this would need to be carried out alongside a protein purification attempt, which may give rise to further technical difficulties.

Although several possibilities exist, the precise cause or causes of the failure of this technique were not resolved or determined by considerable trouble-shooting and modification to technique. Therefore in the interests of moving the research forward, and in view of time and resources already spent on this technique, it was decided to utilise limited existing stocks of HLA-DR15 to obtain the proof of principle this research set out to achieve. Generation of proof of principle data is described in chapter 5 below. Although this technique was not successful in purifying soluble HLA class II, an ELISA was successfully developed for the detection of soluble HLA class II. This could potentially be used in other contexts. For example, there is evidence to suggest shedding of HLA-DR from monocytes in sepsis [192, 193]. This ELISA could potentially be applied to detection of soluble HLA-DR in these cases.

4.5 Conclusions

The work described in this chapter is mainly methodological and therefore generates few conclusions, however it can be concluded that an HLA-DR based ELISA has been developed which is able to detect HLA-DR to 15ng/ml. This ELISA may have the potential to be used in other applications.

5. HLA class II – SAg binding in solution

5.1 Introduction

Early research into superantigens following their initial discovery focussed on the cellular responses. This included techniques such as *in vitro* T cell proliferation assays [194], or *in vivo* studies of pyrogenicity and lethality, usually conducted in rabbits [148, 164, 194, 195]. In particular, mouse fibroblasts transfected with native, mutated or chimeric human HLA class II were initially used to study superantigenic responses [65, 83]. These techniques are still widely used today and provide valuable insights into the potency and lethality of superantigens and their Vβ signatures, in addition to characterisation of the binding sites for superantigens on HLA class II, but do not necessarily allow the detection of superantigens, or determination of their finer biochemical characteristics.

The overall aim of this work was to develop an assay for the detection of superantigens in solution using soluble HLA class II. This required a fundamental proof of principle that HLA class II can be utilised in this way in a cell-free system. Although previously SAgs were generally studied in cell-based systems as described above, more recently cell-free systems such as surface plasmon resonance (SPR), sometimes referred to as BIAcore, have been refined and optimised for the study of superantigens [85, 180]. These have allowed researchers to investigate binding affinities for SAgs to HLA class II and the T cell receptor Vβ region. Numerous HLA class II–SAG complexes and HLA class II-SAg-TcR complexes have also now been crystallised in a cell free environment [24, 32, 51] which have improved the understanding of the structure of SAgs and their binding modes to HLA class II and the TcR, and in turn infer SAg functionality and modes of action.

However, the majority of cell-free systems currently available focus on characterising the biochemical properties of superantigens, rather than detecting the molecules. Detection of superantigens is now typically carried out using PCR, screening bacterial genomes for the presence of superantigen genes [7, 91, 161, 171, 178]. In addition, a few assays have been developed to detect the presence of SAg antibodies in serum, which can be used to infer the presence of SAgs [164, 196]. Previously various ELISAs have been developed to detect individual superantigens, in particular TSST-1 [197-199], however these assays require monoclonal or polyclonal antibodies, and are therefore specific to only one or two SAgs, and are generally restricted to the classical SAgs as very few SAg antibodies are currently commercially available. This restricts the range of superantigens which can be detected. In 2004 Llewelyn *et al* described a new ELISA which utilised the HLA class II molecule as a

general 'antibody' for a range of classical superantigens. This assay also demonstrated the reversal of binding of SAgs to HLA class II using the chelation agent EDTA. The ELISA utilised native HLA-DR and biotinylated SAgs. Detection was achieved with HRP conjugated streptavidin and TMB substrate [66]. This assay provided the proof of principle that HLA class II can be used in this way to detect a range of SAgs, reducing the need for multiple SAg antibodies.

The technique described in this chapter draws on the basic principles from this assay, and develops it further to remove the need to biotinylate SAgs, thus allowing the researcher to detect both classical and novel SAgs in their native states. As the binding of superantigens to HLA class II β chain is zinc dependent [32, 69], the degree of zinc dependency in superantigen binding of HLA class II was tested by zinc addition/chelation (using EDTA).

Although the purification of HLA class II from HLA homozygous B cell lines was unsuccessful, stocks of HLA-DR15 were available from a previous purification by Dr Llewelyn. The staphylococcal superantigen SEA was commercial, whilst streptococcal superantigens SPEA, C and M were recombinantly expressed as described above. SEA was used as a SAg demonstrating both α and β chain binding [64], whilst SPEA was used as a SAg documented to preferentially bind HLA-DQ [67]. SPEC has two β chain binding sites therefore was chosen to demonstrate the zinc dependent nature of β chain binding [24]. SPEM is believed to bind HLA class II β chain zinc dependently with high affinity [26, 58].

Concentration of superantigen used was based on an ELISA developed by Smoot *et al* to detect serum antibodies to SPEL and SPEM in acute rheumatic fever patients [164]. Zinc concentrations used were derived from a published crystallography study [45] using the concentration of superantigen as a starting point. Concentrations of EDTA used were based on decontamination of glassware described by Kay [200].

5.2 – Methods

Buffers used were as described in Table 2.1 above excepting a few specific examples described below.

5.2.1 Detection of superantigens using soluble HLA-DR in an ELISA based assay

ELISAs were carried out as follows (Figure 5.1).

Reagents

Coating Reagent: recombinant superantigens 5µg/ml in carbonate buffer at pH9.0

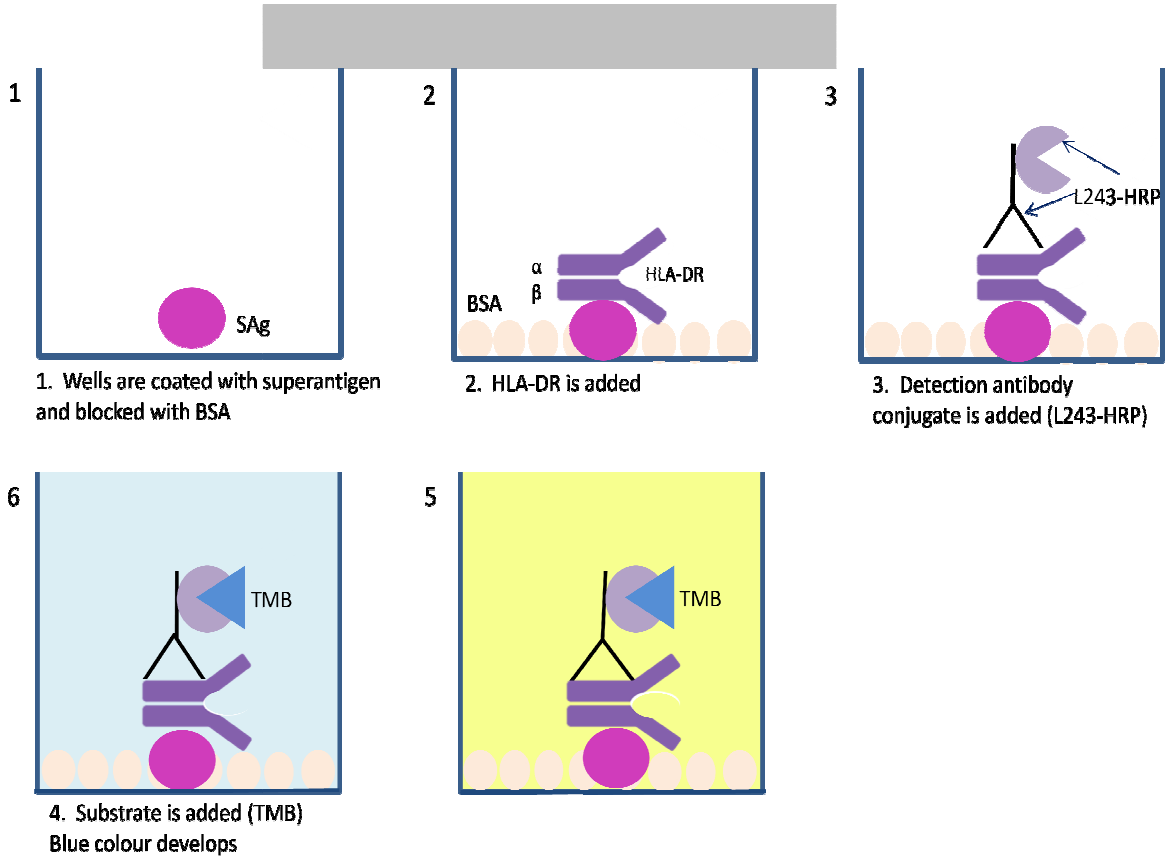
Primary detection reagent: HLA-DR15 prepared in blocking solution +/- zinc/EDTA. Starting concentration 12µg/ml, 8-12 1:2 serial dilutions of this in duplicate.

Second antibody conjugate: L243-HRP used at 1µg/ml prepared in blocking solution +/- zinc/EDTA

Procedure – in experiments with zinc or EDTA, all buffers contained zinc or EDTA as relevant

1. 96 well flat bottomed plates (Corning, UK) were coated overnight at 4°C with 50µl/well 5µg/ml superantigen (Figure 5.1 panel 1)
2. Plates were washed 5 times using 200µl/well wash buffer.
3. Wells were blocked overnight at 4°C using 300µl/well blocking solution.
4. Plates were washed. Duplicates of 50µl HLA-DR standards were added alongside samples and blanks (panel 2)
5. Plates were incubated for 1 hour RT and washed.
6. 50µl/well 1µg/ml detection antibody conjugate was added and incubated for 1 hour RT (panel 3).
7. Plates were washed and 100µl/well TMB substrate added (panel 4)
8. Reactions were stopped after 15 minutes using 100µl/well 2N H_2SO_4
9. Yellow colour was read at 450nm (panel 5) in a Synergy plate reader and results plotted.

Figure 5.1 An ELISA for the detection of superantigens using soluble HLA-DR



5.3 Results

5.3.1 Binding of soluble HLA-DR to superantigens in ELISA based assays

Binding assays were designed with the aim of using soluble HLA-DR to detect superantigens in an ELISA based method. Initial experiments compared binding of SPEC and SEA to HLA-DR and binding of HLA-DR to a preferential DQ binder, SPEA to SPEC.

Figure 5.2a shows the results of a comparison between SPEC and SPEA. HLA-DR binding of both SPEA and SPEC can be detected by ELISA and appears to plateau at the same concentration of HLA-DR (0.38µg/ml). It is noted that binding of HLA-DR to SPEA is easily detectable by ELISA despite documented evidence that SPEA preferentially binds HLA-DQ [67].

Following the success of this assay, the binding assay was repeated for SEA (Figure 5.2b) Results indicated that HLA-DR binding to SEA is detectable at a similar level to SPEA.

As SAg binding to HLA-DR β chain is zinc dependent [32, 69] assays were then extended to include a comparison between binding in the presence and absence of zinc (8µM). Figures 5.3a and 5.3b show binding of HLA-DR to SEA and SPEC in the presence and absence of zinc. In both cases binding without additional zinc was markedly reduced, however a significant level of binding was still detected in the absence of additional zinc. This suggests a level of zinc contamination in buffers, and possible leaching of zinc from glassware [200], as well as a residual level of low affinity binding.

Further from these assays, the effect of zinc chelation using 2mM EDTA was tested. Figure 5.4a appears to indicate that EDTA chelation of zinc has no effect on binding of HLA-DR to SEA. However Figure 5.4b indicates that the presence of EDTA significantly reduces binding of HLA-DR to SPEC.

Following the successful expression and purification of SPEM, and a validation of its superantigenicity, the same binding assay was carried out using SPEM (Figure 5.5). This again indicated a marked reduction of binding upon the addition of EDTA, reinforcing reports that SPEM binds with high affinity to the β chain of HLA-DR [26, 58].

Figure 5.2a HLA-DR binding of SPEC vs SPEA

Points are a mean of duplicate readings from two adjacent wells on one plate +/- 1 standard deviation. Binding of HLA-DR15 to both SPEC and SPEA is easily detectable by ELISA, although binding to SPEA is reduced compared to SPEC.

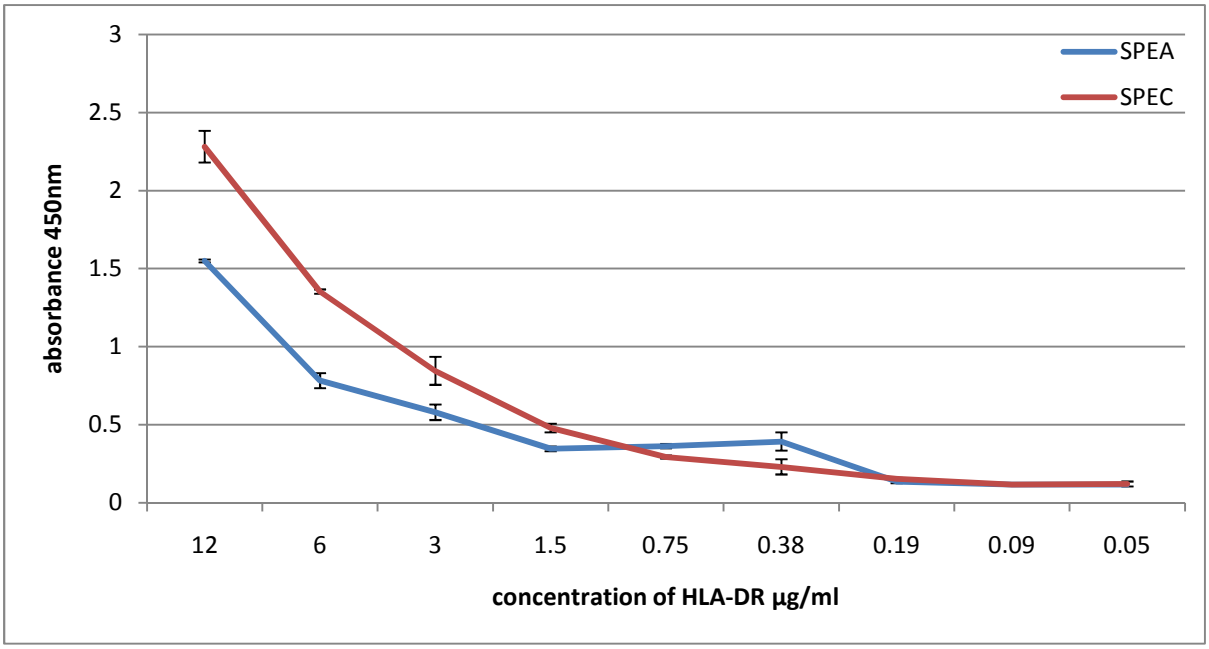


Figure 5.2b SEA binding to HLA-DR

Points are a mean of duplicate readings from two adjacent wells on one plate +/- 1 standard deviation. Binding of HLA-DR15 to SEA is detectable by ELISA at a similar level to SPEA.

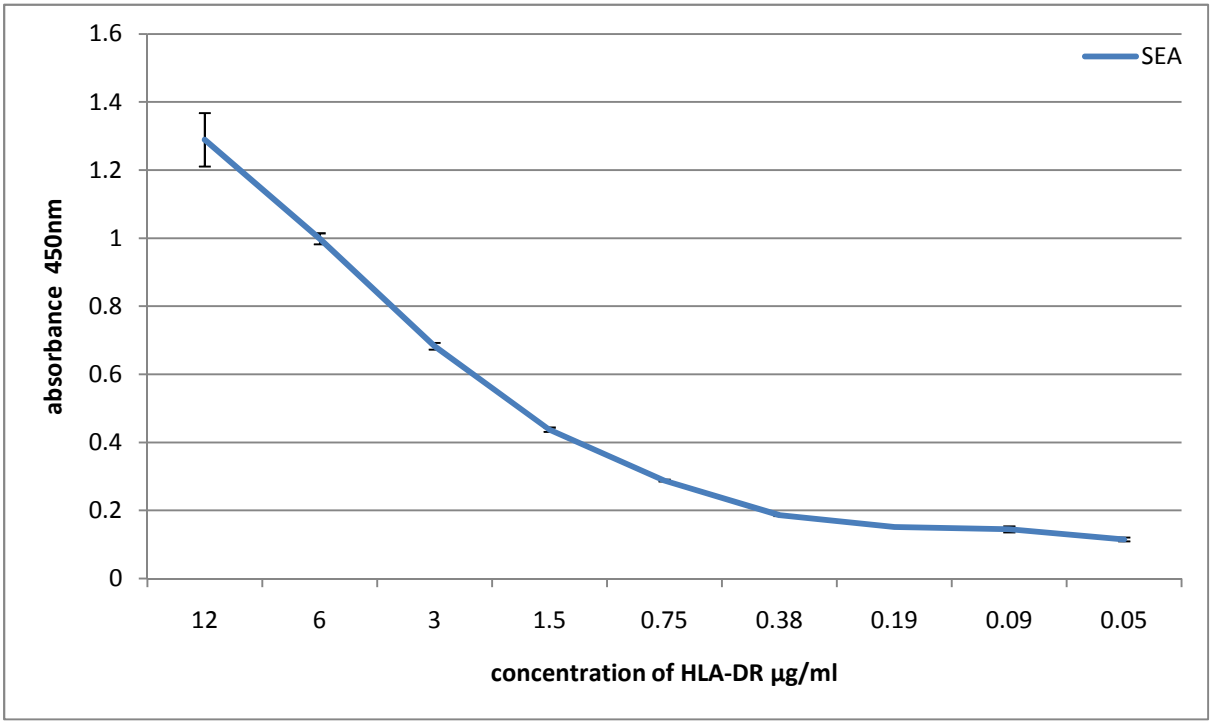


Figure 5.3a HLA-DR binding to SEA in the presence and absence of zinc

Points are a mean of duplicate readings from two adjacent wells on one plate +/- 1 standard deviation. Binding of HLA-DR to SEA is markedly reduced in the absence of zinc.

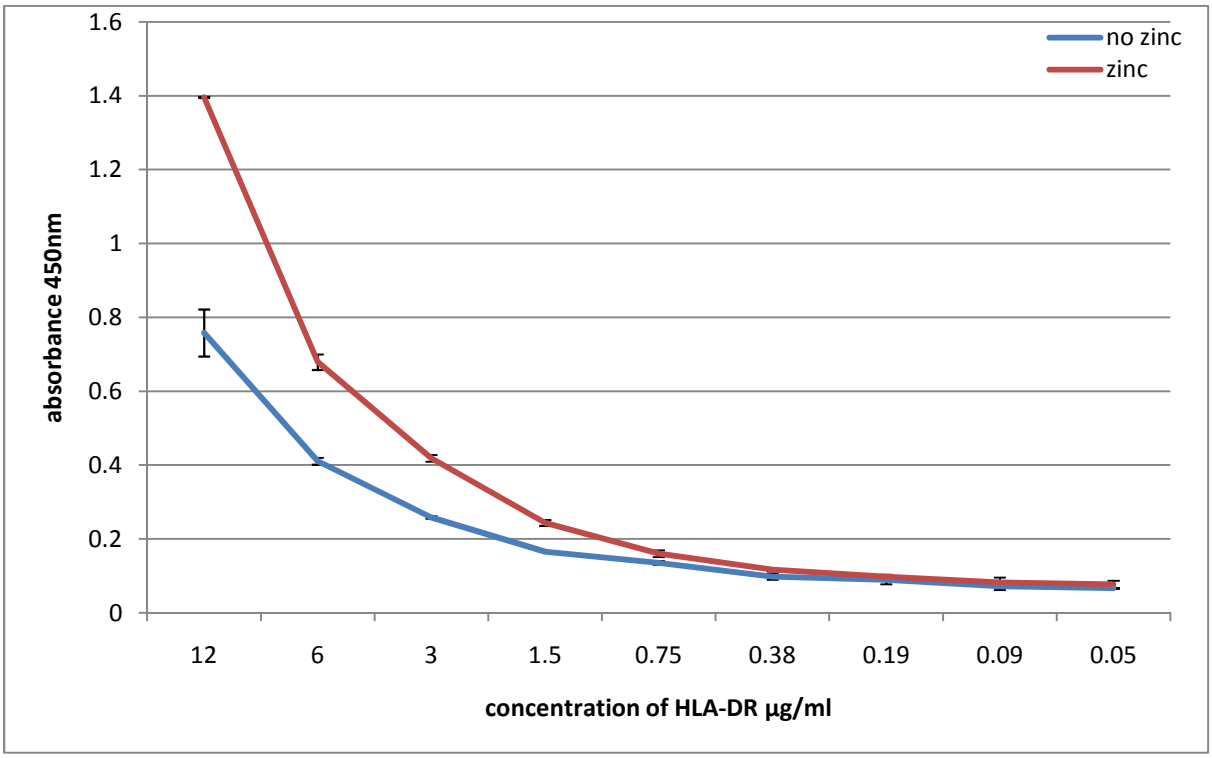


Figure 5.3b HLA-DR binding to SPEC in the presence and absence of zinc

Points are a mean of duplicate readings from two adjacent wells on one plate +/- 1 standard deviation. Binding of HLA-DR to SPEC is markedly reduced in the absence of zinc

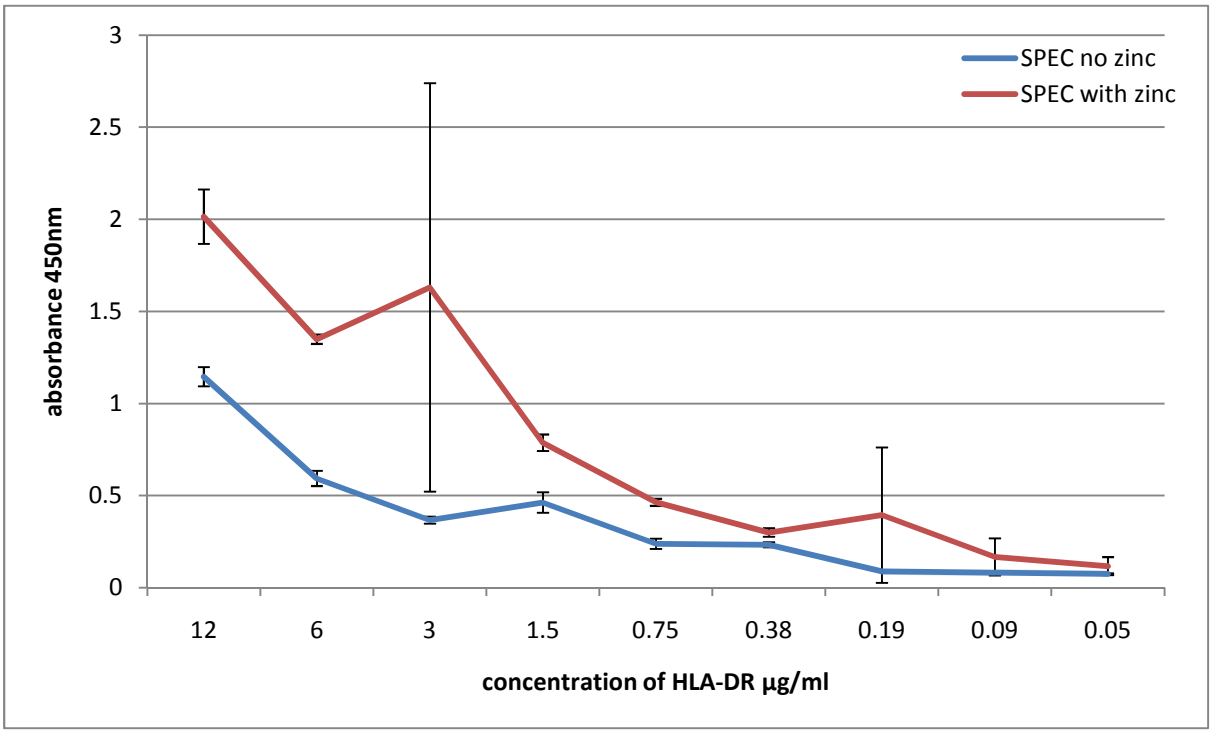


Figure 5.4a Binding of HLA-DR to SEA with zinc buffers and EDTA buffers

Points are a mean of duplicate readings from two adjacent wells on one plate +/- 1 standard deviation. The presence of EDTA appears to have no detectable effect on the binding of HLA-DR to SEA.

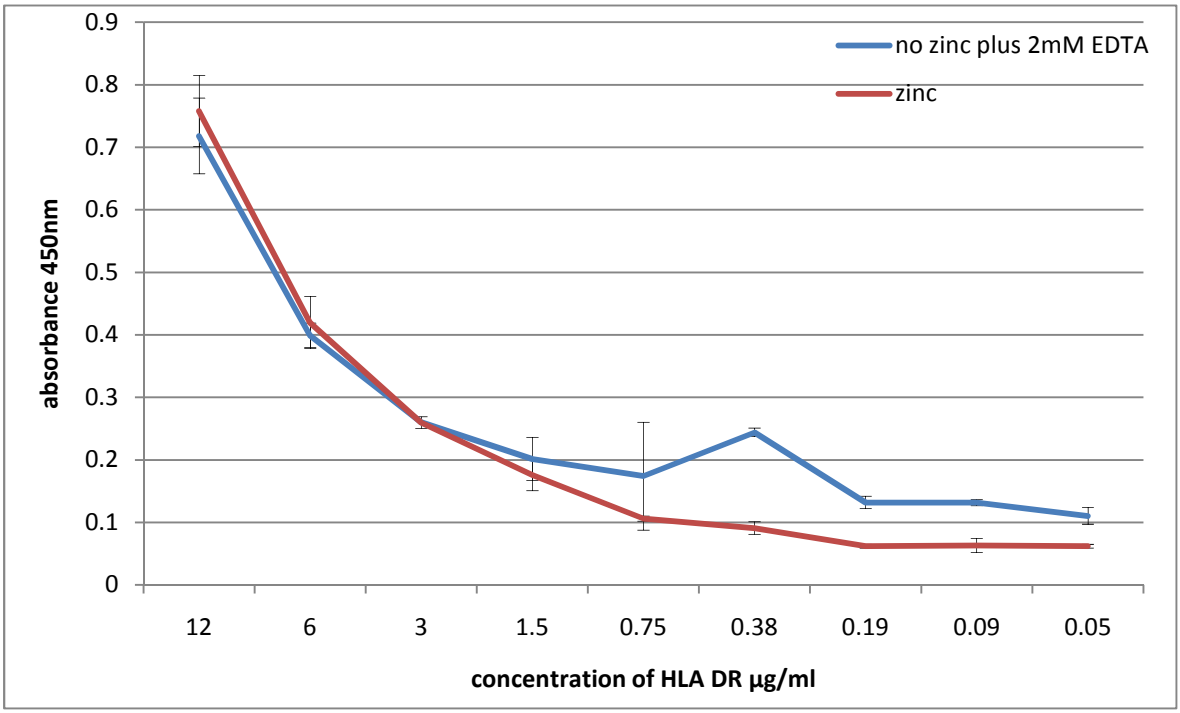


Figure 5.4b Binding of HLA-DR to SPEC with zinc and EDTA

Points are a mean of duplicate readings from two adjacent wells on one plate +/- 1 standard deviation. The presence of EDTA markedly reduces detectable binding of HLA-DR to SPEC compared to buffers containing zinc.

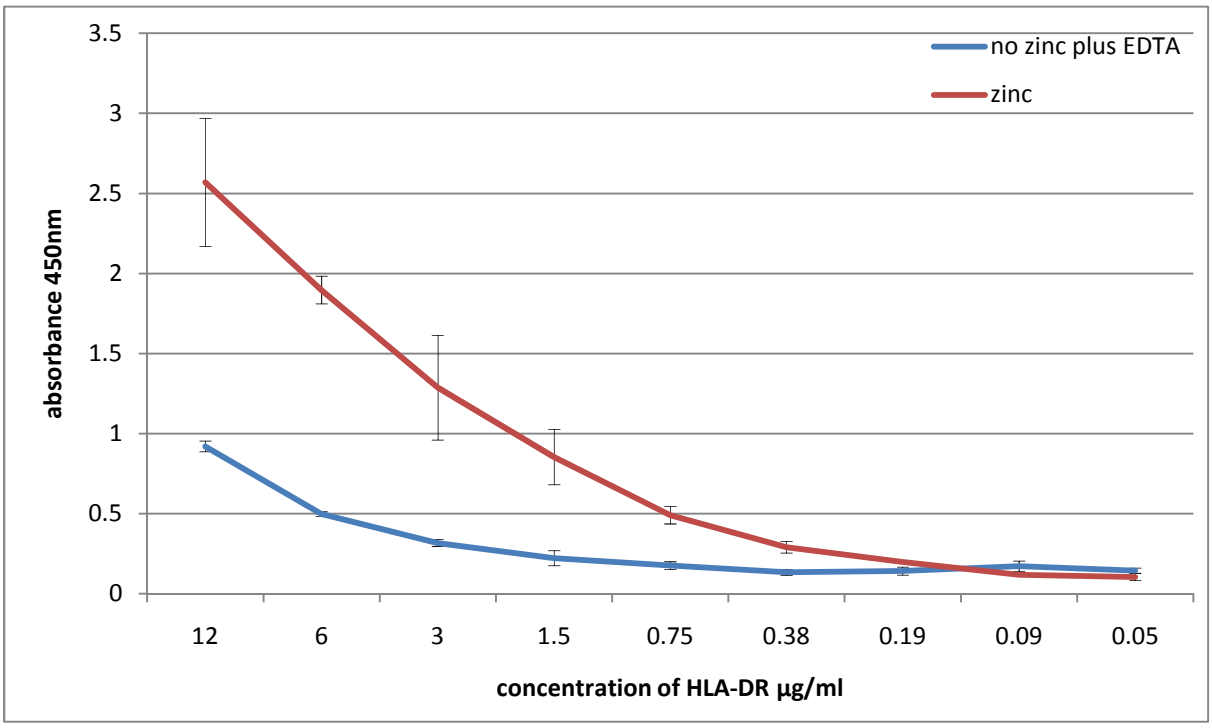
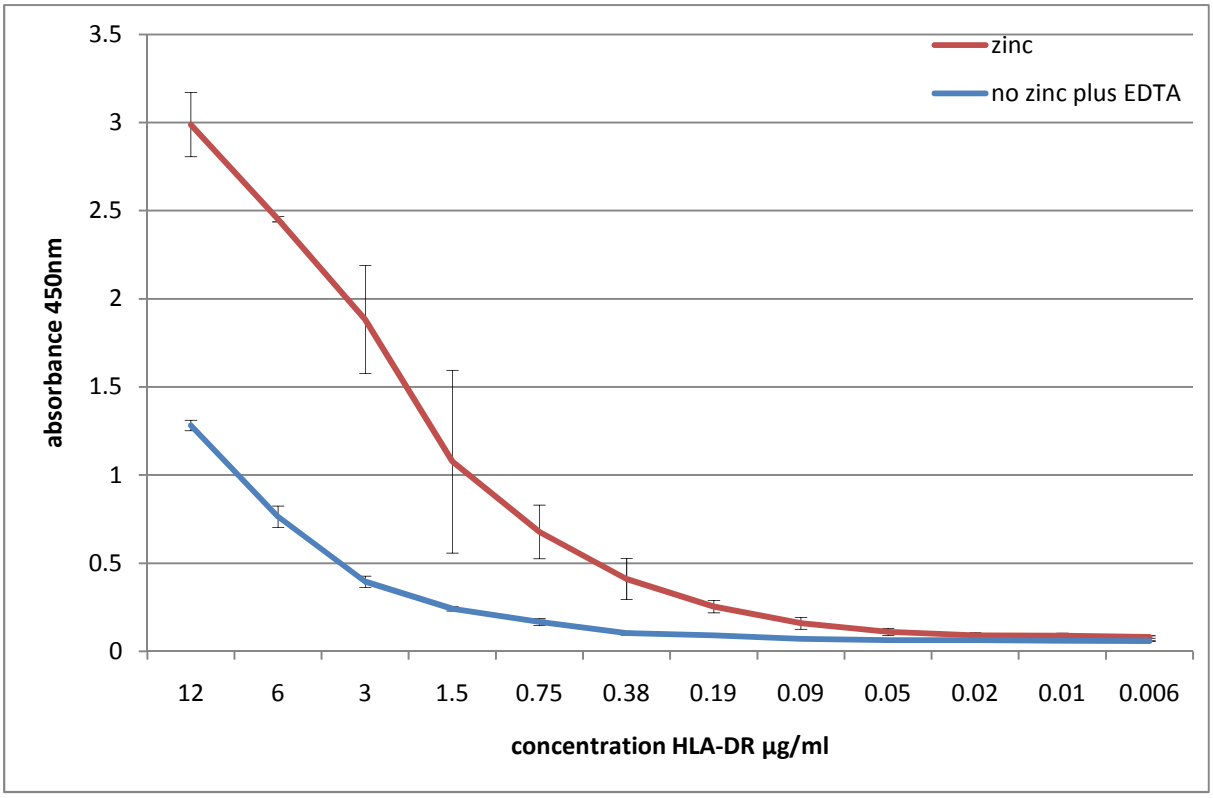


Figure 5.4c Binding of HLA-DR to SPEM with zinc and EDTA

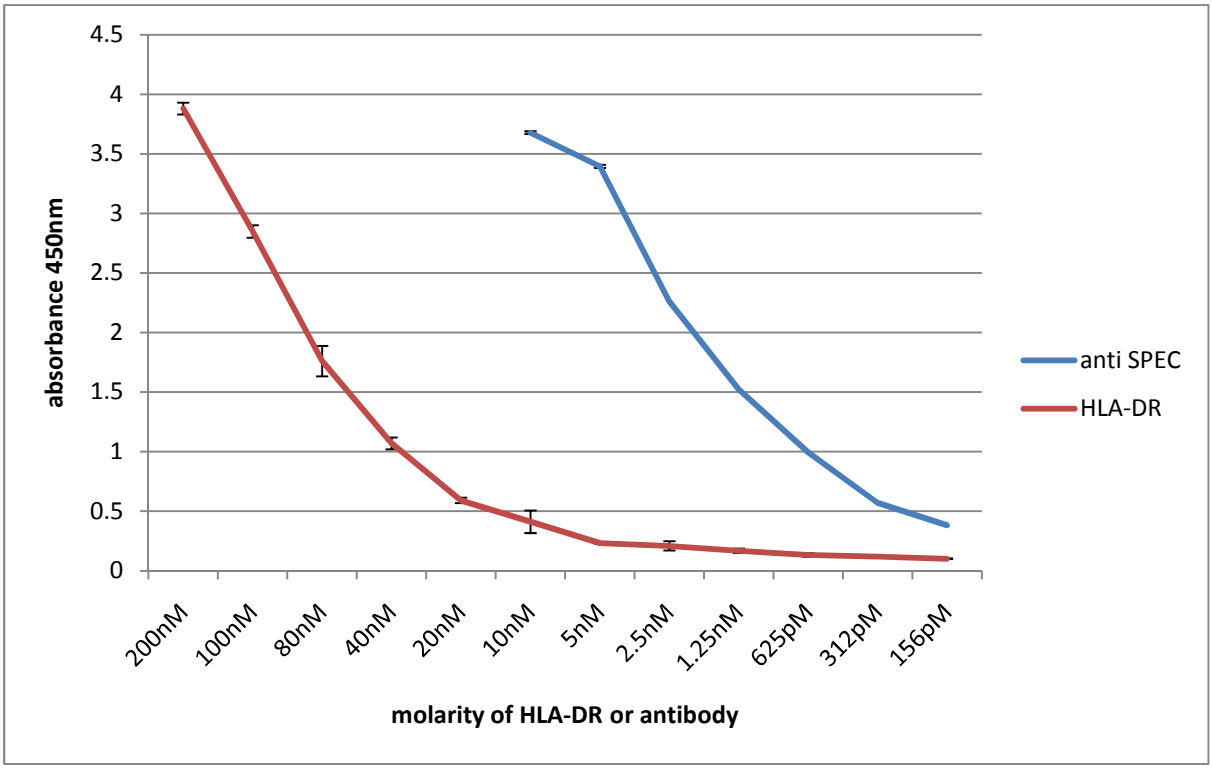
Points are a mean of duplicate readings from two adjacent wells on one plate +/- 1 standard deviation. The presence of EDTA markedly reduces detectable binding of HLA-DR to SPEM compared to buffers containing zinc.



Finally, a comparison was made between the binding of HLA-DR and the binding of anti-SPEC monoclonal antibody. Plates were coated with SPEC and probed with either anti SPEC antibody (Toxin Technology, Sarasota, FL) or HLA-DR in a series of decreasing concentrations identical to the HLA-DR standard curve described in earlier assays in this chapter. Figure 5.5 shows binding of SPEC to anti-SPEC is greater than binding to HLA-DR. The curve was shorter for anti-SPEC antibody as absorbances for concentration of antibody above 10nM were over the scale achievable using the KC4 plate reader. However, although binding of SPEC to its antibody is greater than to HLA-DR, a comparable level of binding is seen with HLA-DR, demonstrating that although the affinity may be lower, specificity is sufficient to detect SAg.

Figure 5.5 – Comparison of SPEC binding to anti-SPEC antibody and HLA-DR

Showing a greater level of binding with anti-SPEC antibody than with HLA-DR, but comparable curves and sufficient specificity of HLA-DR. Points shown are a mean of duplicate readings from two adjacent wells on one plate +/- 1 standard deviation.



5.4 Discussion

The overall aim of this work was to develop an assay for the detection of a range of classical and novel SAgs in solution utilising soluble HLA-DR as a capture molecule. Despite the considerable difficulties generating HLA class II described in Chapter 4 above, successful prototypic binding assays have been performed. Soluble HLA-DR15 (previously purified by above method by Dr Martin Llewelyn) was used with recombinant and commercially available SAgs in a number of ELISA based assays. Initial assays indicated a predicted high level of binding to SPEC, (Figure 5.2a) which contains two β chain binding sites and no α chain site [24]. Binding of HLA-DR to SEA was easily detectable at a lower level than for SPEC, (Figure 5.2b) which may indicate a lower binding affinity of SEA to HLA-DR. The most interesting result was that binding of HLA-DR to SPEA was detectable at a similar level to SEA, (Figure 5.2a) despite published data indicating that SPEA preferentially binds HLA-DQ [67]. Following initial assays, the effect of additional zinc in solution was investigated. In all cases additional zinc resulted in a higher level of HLA-DR binding to superantigens than buffers containing no zinc (Figures 5.3a and b), supporting evidence that HLA-DR β chain binding of SAgs is zinc dependent [45]. However a residual level of binding without zinc was observed, suggesting possible zinc contamination and leaching of zinc from glassware. This may also be due to lower affinity binding through the α chain binding site.

Therefore the effect of EDTA chelation of zinc was also investigated. Interestingly here EDTA acting as a powerful chelating agent [200] did not appear to affect binding of SEA to HLA-DR (Figure 5.4a). This could indicate the importance of α chain binding by SEA, and provides evidence that HLA-DR could be used as a capture molecule for a wide range of superantigens. However, EDTA chelation of zinc reduced the binding of HLA-DR to SPEC (Figure 5.4b)**.** This could indicate the possibility of using EDTA as a reversal agent for use in other future assays, both of this type and other assays described in future work. Finally, Figure 5.4c demonstrates that this assay also applies to the novel superantigen SPEM, and that EDTA chelation reduces binding of SPEM to HLA class II, demonstrating the wide range of superantigens this technique could apply to. Finally, a comparison of binding of SPEC to anti-SPEC antibody and HLA-DR showed that although greater affinity of antibody for SPEC than HLA-DR for SPEC was apparent, the specificity of HLA-DR for SPEC was sufficient to see a comparable titration curve to that of antibody. This highlights how by using HLA-DR or class II based ligands as capture molecules the user may have to sacrifice high affinity binding for broad specificity, however modifications could be made to the structure of HLA class II to improve affinity.

It can be seen from these data that a new assay for the detection of superantigens drawing its basic principles from existing assays has been developed. This has potential in a laboratory setting to detect superantigens in bacterial lysates or biological samples with relative ease, and

without the need to biotinylate key reagents. Importantly, this assay is able to detect novel superantigens, for which there are no commercially available antibodies. Modifications to the technique, such as establishing a standard curve in serum, should determine the ability to detect superantigens in clinical samples, and therefore determine the future implications of this technique. A structure based design approach could also be used to design HLA class II based peptides with broad specificity and high affinity for a range of superantigens.

5.5 Conclusions

This chapter has provided some preliminary evidence and proof of principle that soluble HLA class II can be used to detect bacterial superantigens in an ELISA based assay. The following can be concluded:

- Soluble HLA class II can be used to detect a range of bacterial superantigens
- This range of SAgs includes preferential HLA-DQ binders (SPEA) and SAgs which utilise the low affinity HLA class II binding site (SEA.)
- Addition of zinc increases binding of all superantigens studied
- EDTA chelation of zinc reduces binding of all but one SAg.

6. Superantigenicity of SPEC, SPEL and SPEM

6.1 Introduction

The work described in this chapter aimed to investigate firstly the superantigenicity of SPEC, L and M in terms of HLA class II binding mechanisms and secondly the superantigenicity of SPEM in terms of Vβ responses, particularly between HLA similar donors in order to determine whether the differences observed between Vβ responses to SPEM between donors in chapter 3 could relate to HLA class II differences.

SPEC has been well characterised in terms of its interactions with HLA class II. SPEC is a Group IV superantigen, grouped with other streptococcal superantigens according to the most recent grouping system (see Figure and Table 1.3 in Chapter 1) which utilise the HLA-DR zinc dependent β chain binding site [24, 26, 32, 58, 63]. SPEC contains 2 β chain binding sites [7, 24, 63]. Recently Kasper *et al* identified a previously undescribed low affinity 'interface' in SPEC similar to that found in SEB, which may be utilised in the binding of SPEC to HLA-DQ [56]. SPEC is one of the most potent superantigens thus identified, inducing half maximal proliferation at 0.1pg/ml [7]. SPEC has been demonstrated to have a dissociation constant for peptide loaded HLA class II of $\leq 0.1\mu M$ [24].

Several studies have described the Vβ response to SPEC and have indicated that the Vβs responding to SPEC are 2.1, 3.2, 12.5 and 15.1 [7, 58] [63]. Additionally it has been demonstrated that the side chains of residues present in TcR Vβ regions are heavily involved in SPEC binding. The inference being that mutations in these sites will reduce SPEC binding. Contacts with HLA class II loaded peptide also seem to be important in SPEC binding [26, 75, 201]. It has not been determined whether or not there is a hierarchy of Vβ responses to SPEC.

SPEL and M are among the more recently described streptococcal superantigens. The nature of their interaction with HLA class II is less well established. Based on sequence homology and postulated structural similarities SPEL and SPEM have also been grouped into Group IV (Figure and Table 1.3 above) [26], implicating zinc dependent β chain binding of HLA class II. The zinc dependent binding of SPEM has been confirmed by the work presented in Chapter 5 of this thesis. No characterisation of the binding affinities of SPEL and M exists [58, 164]. Vβ responses to SPEL and SPEM have been described in two instances, using PCR [97] and flow cytometry [164]. These revealed a Vβ signature for both toxins of Vβs 1, 5.1 and 23. Hierarchical Vβ responses to SPEL and SPEM are not characterised [164]. During the course of this work (Chapter 3) differences in the hierarchy and magnitude of Vβ responses to SPEL and SPEM between individuals were noted, particularly with SPEM. The classification of these

superantigens into the same group as SPEC, as well as existing evidence for the effect HLA polymorphisms have on SPEC responses [67] leads to the hypothesis that HLA polymorphisms may also influence responses to SPEL and SPEM. Further, evidence that SPEL and M bind HLA-DR in a zinc dependent manner [7, 58, 164] suggests that polymorphisms in HLA-DR β chain may be of particular importance with these superantigens. Table 1.2 in Chapter 1 shows key residues for SAg binding identified in the HLA-DR β chain in various crystallographic studies [32, 45, 64, 69].

The work described in this chapter aims to further characterise the interactions of SPEC, SPEL and SPEM with HLA class II, establish the hierarchy of Vβ specific responses to these superantigens, and explore the potential for HLA class II polymorphisms to explain inter-individual difference in Vβ specific response.

6.2 Methods

The following methods were used to obtain the results described in this chapter.

6.2.1 Conjugation of SAgs and antibodies with PE and FITC

Recombinantly expressed streptococcal superantigens C, L and M (as described in Chapter 3 above) were conjugated to recombinant phycoerythrin using the LightningLink RPE kit according to manufacturer's instructions (Innova Biosciences, UK). Briefly 60µg SAg was incubated with lyophilised R-PE and LightningLink modifier reagent for 3 hours at room temperature. Reactions were quenched with LightningLink quenching reagent and conjugated SAgs stored at 4°C in the dark.

L243 (anti-HLA-DR α chain, from supernatant of HB95 hybridoma cell line, LGC Promochem UK) and TDR31.1 (anti-class II β chain, Ancell Alexis Corp) were conjugated with fluoroscein isothiocyanate (FITC) using the Fluoro-Trap Fluoroscein labelling kit (Innova Biosciences, UK). 100µg antibody was incubated with lyophilised fluoroscein and Fluoro-Trap reaction buffer for 3 hours at room temperature. Reactions were quenched for 1 hour at room temperature using Fluoro-Trap quenching reagent and excess dye removed using Fluoro-Trap columns. Neutralising solution was added and conjugated antibody stored at 4°C in the dark.

6.2.2 Flow cytometric analysis of SAg-HLA class II binding

The WT51 B cell line expressing HLA-DR4 and DQ8 was cultured as described in chapter 2. To assess SAg binding to WT51 cells, fresh cells were harvested by centrifugation on the day of each experiment. Aliquots of 5 x 10^5 cells/per sample were stained on ice in the dark for 45 minutes with varying concentrations of SAg-PE or anti class II antibody-FITC. Samples were

washed twice by centrifugation at 1000 x rpm in FACS buffer with zinc (see Table 2.1 above) and analysed by flow cytometry. In experiments to determine the ability of antibodies to HLA class II or unconjugated superantigen to block binding of conjugated superantigen cells were pre-incubated with either antibody or unconjugated SAg for 45 minutes on ice before being stained as above.

6.2.3 HLA typing and grouping of HLA types

Ethical approval was obtained from the BSMS Research Governance and Ethics committee to HLA type healthy donors and investigate SAg responses related to HLA type. Following recruitment, 5ml peripheral blood was drawn from 10 donors using the BD Vacutainer system and sent to the HLA Informatics group at the Anthony Nolan Trust for HLA typing in the *DRB1, DQB1* and *DQA1* genes. 5 of 10 donors were grouped into two groups based on similarities between HLA-DR types.

Two donors were homozygous for *DRB1*04*. Although their allelic variants differed (e.g. LLEM4 was *DRB1*0404 DRB1*0403*), amino acids at the important sites highlighted in Table 1.2 above were identical. Therefore these donors grouped together as Group 1.

One other donor was homozygous for *DRB1*0701*. This donor was grouped with two heterozygous donors who were *DRB1*1101 DRB1*1301* and *DRB1*1101 DRB1*0701*. *DRB1*0701, DRB1*1101* and *DRB1*1301* are identical in the amino acids present at sites considered. These donors were designated Group 2. Groups 1 and 2 differed in one residue within these important sites. Position 70 was glutamine (Q) in Group 1 and aspartic acid (D) in Group 2. These residues differ in charge in their side chains (D is positive, Q is neutral); therefore this may impact upon SAg binding [202]. When considering the overall sequence of the 21 bases surrounding these key residues (bases 66-86), Group 2 in particular differed in several bases. However most of these differences are fairly inconsequential. For example position 67 contained either an isoleucine or phenylalanine residue in this group, but as both these residues are hydrophobic [202] is it unlikely this will have influenced SAgs binding to any great extent. Appendix 3 shows a table of the differences between these 21 bases in the five different alleles used. Five other heterozygous donors could not be used as amino acids at these important sites between their HLA types did not match.

Following grouping of donors based on HLA-DR similarities, peripheral blood was taken from donors and used in Vβ proliferation experiments with SPEM as described in chapter 2 above, incubating cells with toxin for 48 hours. This was repeated three times for each donor and the results analysed by flow cytometry.

6.3 Results

6.3.1 Verification of superantigenicity of PE conjugated SAgs

To determine whether fluorochrome-conjugation of the SAgs had had any impact on their function Vβ specific proliferation of PBMCs was assessed by flow cytometry. SPEC-PE showed a clear skewing to Vβ12 as expected [7] compared to the PHA response, although percentages were lower than in previous SPEC Vβ proliferations described in Chapter 3. (Figure 6.1a).

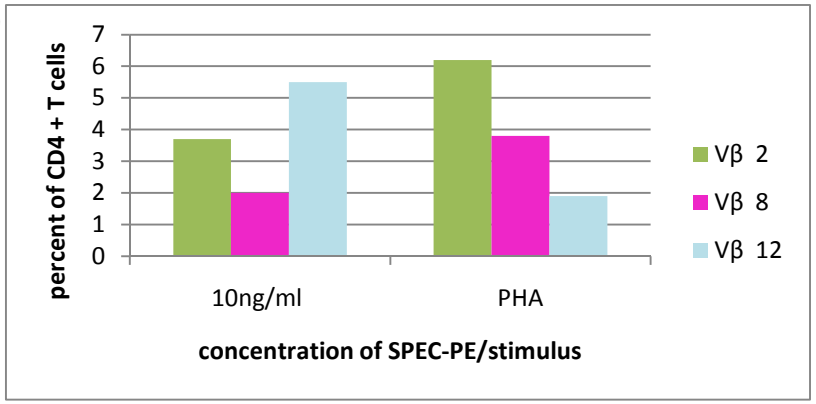
SPEL-PE demonstrated a Vβ1 specific response compared to the PHA response. Other Vβs studied also expanded, including Vβ8, but to a lesser extent than Vβ1 (Figure 6.1b). Expansion in Vβ1 was similar to that observed previously in Chapter 3 although at a higher overall proportion (Figure 3.7b)

Finally SPEM-PE demonstrated expansions in Vβ1 primarily and Vβ5.1 to a lesser extent (Figure 6.1c). This is consistent with findings described earlier in chapter 3.

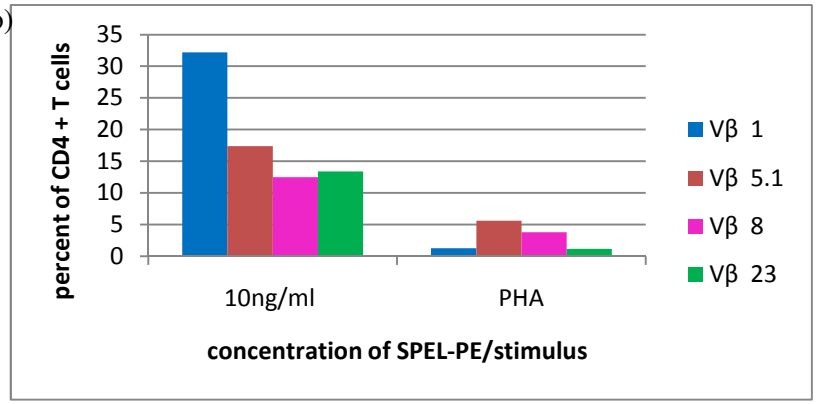
Figure 6.1 Percent of CD4+ T cells bearing specific Vβ regions in response to SAg-PE stimulation

Results are for one donor in a single experiment following stimulation of PBMCs with SPEC, SPEL or SPEM for 48hrs. a) SPEC is documented to cause an expansion in Vβs 2.1, 3.2, 12.5 and 15.1 [7, 58]. Expansion in Vβ12 is seen at 10ng/ml compared to the PHA response. b) Showing an expected expansion in Vβs 1 and to a lesser 5.1 and 23 in response to SPEL-PE. c) Showing expected expansions in Vβs 1, 5.1 and 23 in response to SPEM-PE.

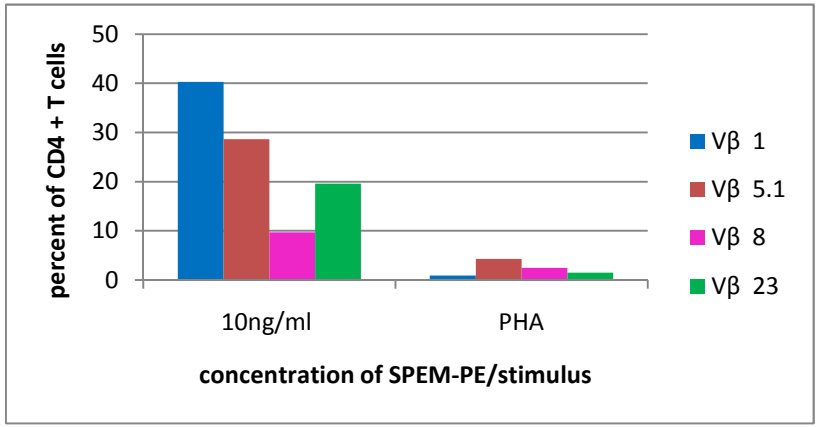
a)



b)



c)



6.3.2 Binding of SAg-PE to HLA homozygous B cell line WT51

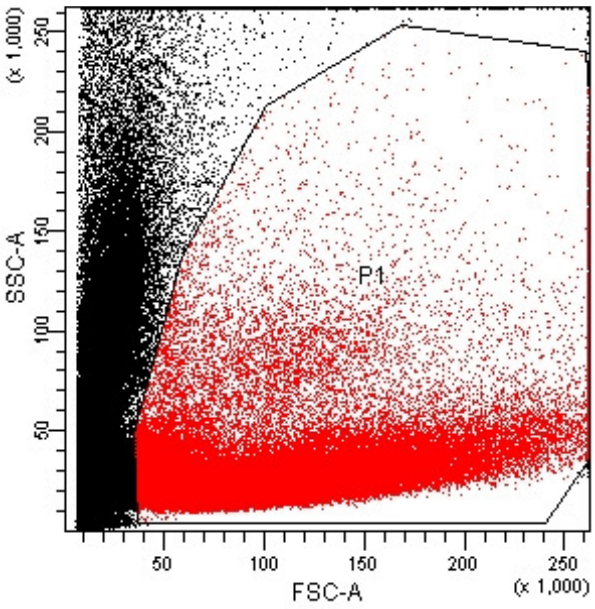
Vβ validation of conjugated SAg indicated showed some confusing results but still indicated Vβ specific proliferation in response to SAg compared to the PHA response. Therefore PE conjugated SAgs were used in various flow cytometric assays to characterise binding to HLA class II. HLA class II expression on WT51 cells was also assessed using FITC conjugated L243 and TDR31.1. In all flow cytometric binding assays gates were used to exclude cell debris (population P1 shown in red Figure 6.2a). Histograms showing geometric means for PE and FITC were generated based on population 1 (P1, Figure 6.2b). This gating strategy was employed for all flow cytometric experiments including WT51, SAg-PE and anti class II antibodies.

Figure 6.2 A typical gate used in B cell line –SAg binding assays and histograms generated

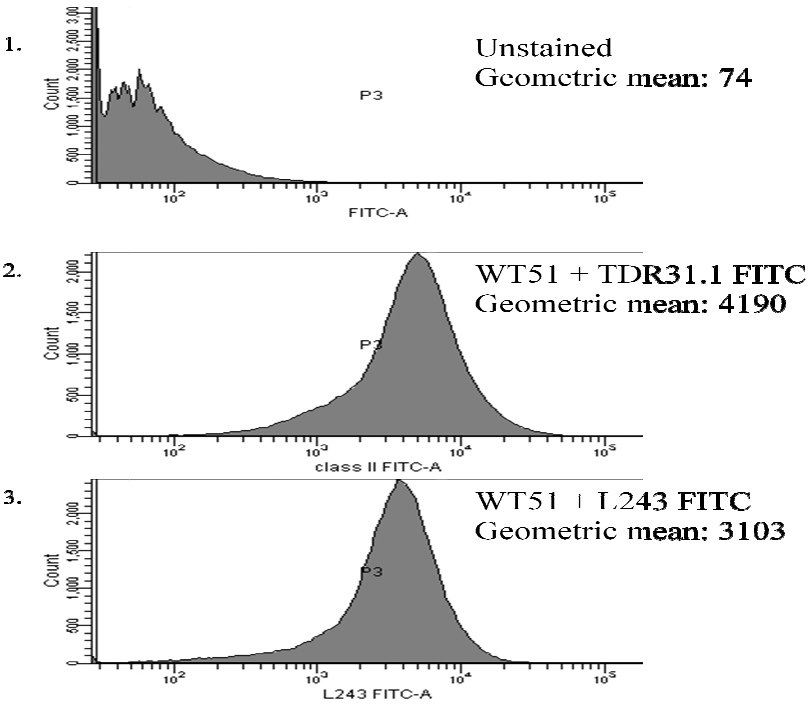
a) Dotplot of WT51 cells showing population 1 (P1) in red, and gate excluding cell debris.

b) Histograms were generated showing P1 only and geometric means calculated from these. This example shows FITC geometric means for unstained WT51 (1) WT51 + TDR31.1 (2) and WT51 + L243 (3). Staining was carried out on ice for 40 minutes.

a)



b)



6.3.2a HLA class II expression on WT51 cells and binding of SAgs to HLA class II

HLA class II expression on WT51 cells was assessed in triplicate using anti-class II antibodies L243 (anti DR α chain) and TDR31.1 (anti class II β chain) conjugated to FITC. An example of a histogram generated from these experiments is seen in Figure 6.2b. Geometric means were converted to multiples of unstained geometric means (Figure 6.3). This showed a population shift from unstained cells to those stained with both antibodies, indicating expression of HLA class II on WT51 cells as expected. TDR31.1 binding appeared to be greater than L243 expression, however as this antibody binds pan class II β chain and therefore includes HLA-DQ and DP, whilst L243 only binds HLA-DR α chain this is not unexpected. However the difference between the two antibodies is not statistically significant ($P = 0.1$), possibly indicating a very similar level of binding of both antibodies overall.

Following this verification of HLA class II expression on WT51 cells, SAg-PE-HLA class II binding was assessed. Initially 10μg/ml SAg-PE was used for each toxin. 5×10^5 cells were stained with SAg-PE using FACS buffer with no additional zinc. However this revealed no population shift for any toxin and therefore indicated no binding. As SPEC has been demonstrated previously to bind HLA-DR β chain in a zinc dependent manner, [24] whilst zinc addition has been shown to increase binding of SPEM to soluble HLA class II in the course of this work, zinc was added to the buffers. As before with binding assays described in chapter 5, the addition of zinc increased binding. SAg-PE binding to HLA class II was detected with all toxins and demonstrated by a population shift compared to unstained cells (Figure 6.4).

Figure 6.3 Expression of HLA class II on WT51 cells

Ratio of geometric means to unstained GM showing more fluorescence on stained cells, indicating class II expression. TDR31.1 binding appears to be greater than L243 binding but this is not statistically significant (P= 0.1). Points shown are a mean of triplicate readings from three experiments using the same batch of WT51 cells +/- 1 standard deviation. Staining was carried out on ice for 40 minutes in the dark.

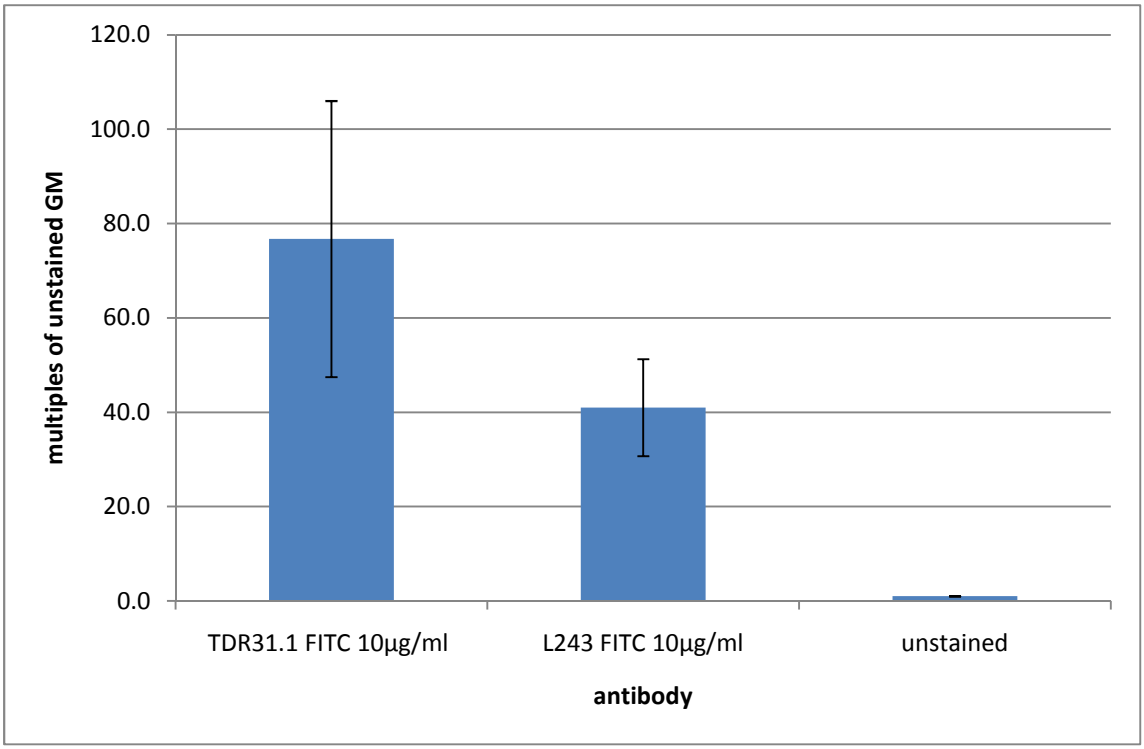
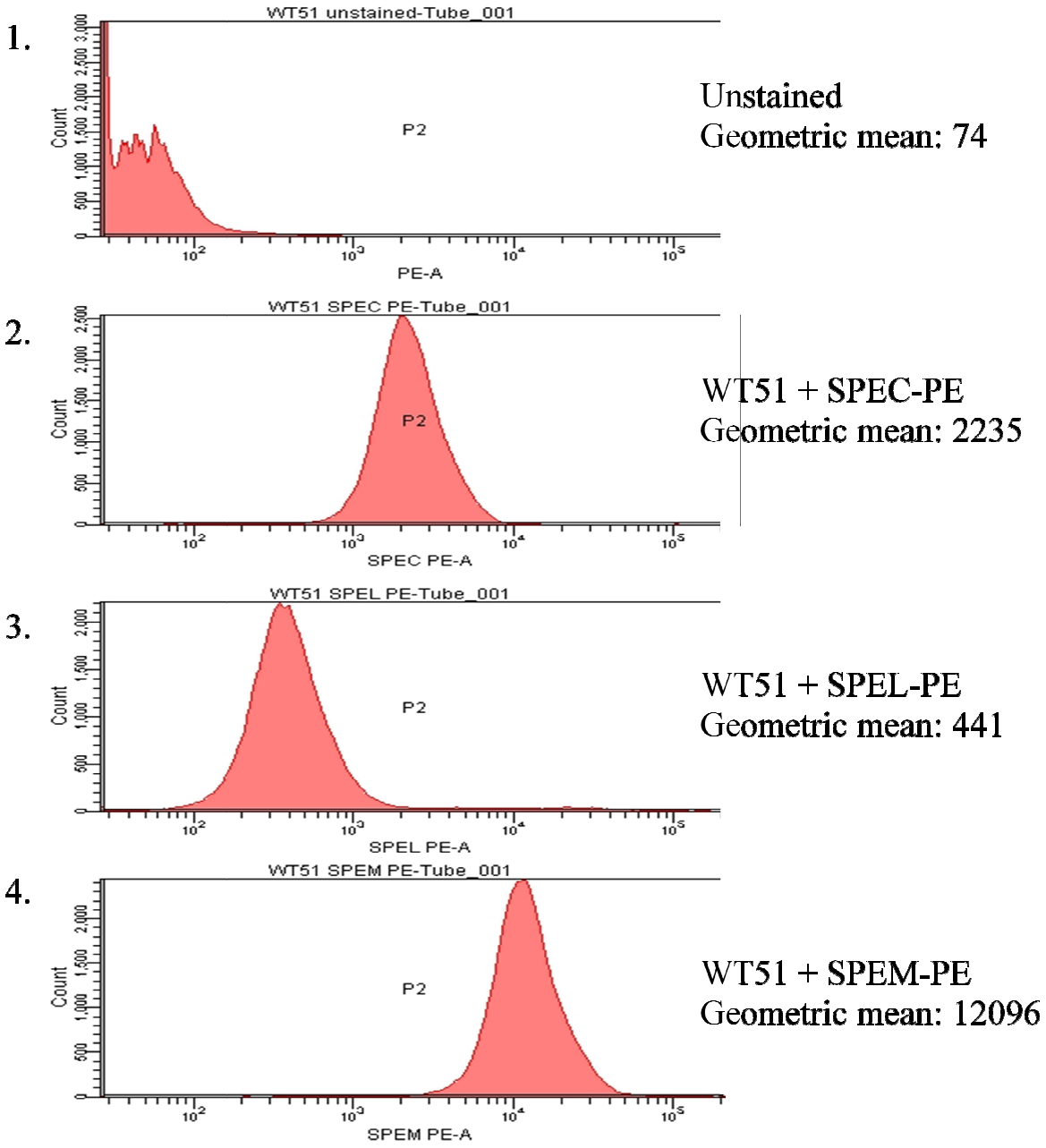


Figure 6.4 Binding of SAg-PE to HLA class II with zinc

Fluorescence histograms of unstained WT51 cells (1) vs WT51 cells stained with SPEC-PE (2), SPEL-PE (3) and SPEM-PE (4) are shown. Histograms shown are the result of a single experiment and representative of other experiments. Staining was carried out on ice for 40 minutes in the dark.



6.3.2b Titration of SAg

Having demonstrated that a) HLA class II expression can be demonstrated on WT51 cells, and b) that PE conjugated SAgs will bind HLA class II when zinc is present, titration curves using 10 fold dilutions of SAg-PE were established for each of the three toxins (Figures 6.5, 6.6 and 6.7). Results were expressed as a ratio of the geometric mean fluorescence of stained to unstained cells. The lowest concentration at which binding was detectable was 1µg/ml for SPEC and SPEM, showing P values of 0.02 and 0.002 respectively. Detectable binding of SPEL was not statistically significant even at the highest concentration of toxin (P = 0.07). From this it was decided to use 1µg/ml SPEM-PE and 10µg/ml SPEL-PE in competition binding assays described below. 5µg/ml SPEC-PE was used for further assays, chosen from these results as a midpoint between a very high level of binding (10µg/ml) and a statistically significant level of binding (1µg/ml).

Figure 6.5 Titration curve for SPEC-PE

As concentration of PE stained toxin decreases so too does fluorescence. The P value at 1µg/ml compared to unstained is statistically significant at 0.02. Points shown are a mean of triplicate readings from three separate experiments using the same batch of WT51 cells +/- 1 standard deviation. Staining of WT51 cells was carried out for 40 minutes on ice.

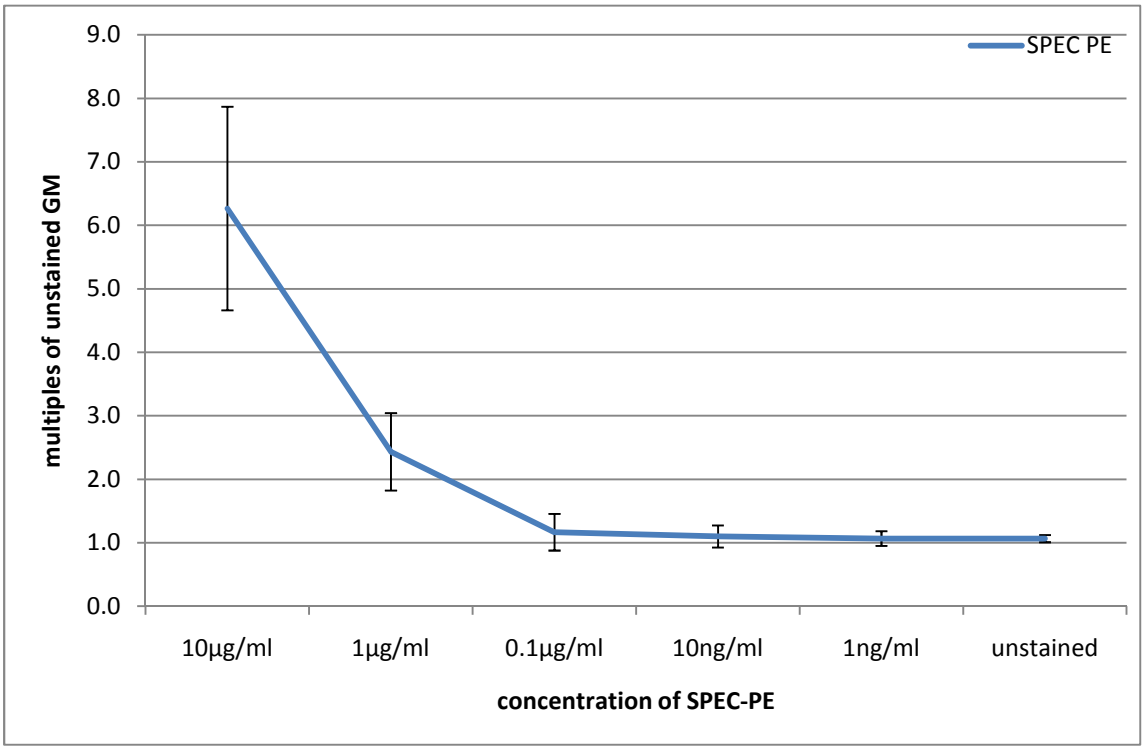


Figure 6.6 Titration curve for SPEL-PE

The degree of detectable binding of SPEL-PE to HLA class II is lower than for SPEC-PE or SPEM-PE and varies greatly between experiments. The results are not statistically significant (P=0.07 at 10µg/ml). Points shown are a mean of triplicate readings from three separate experiments using the same batch of WT51 cells +/- 1 standard deviation. Staining was carried out for 40 minutes on ice in the dark.

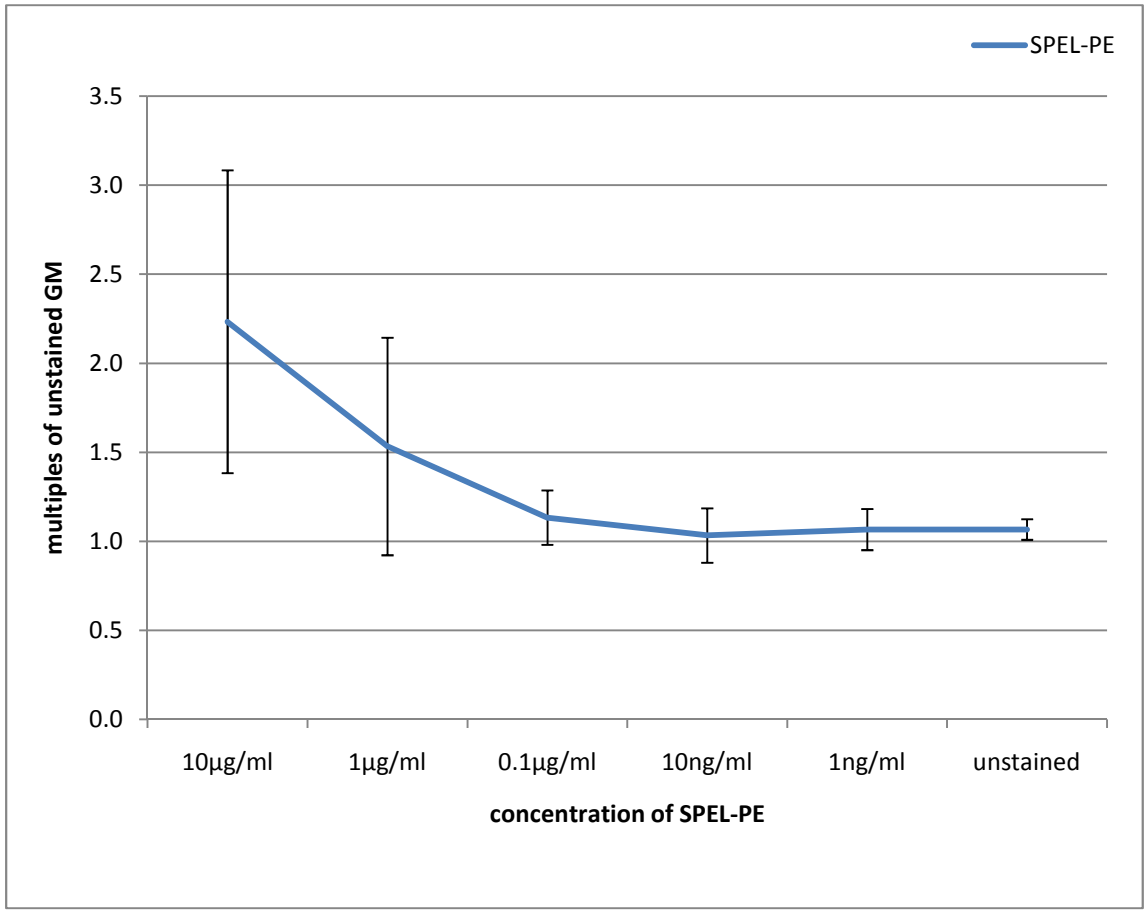
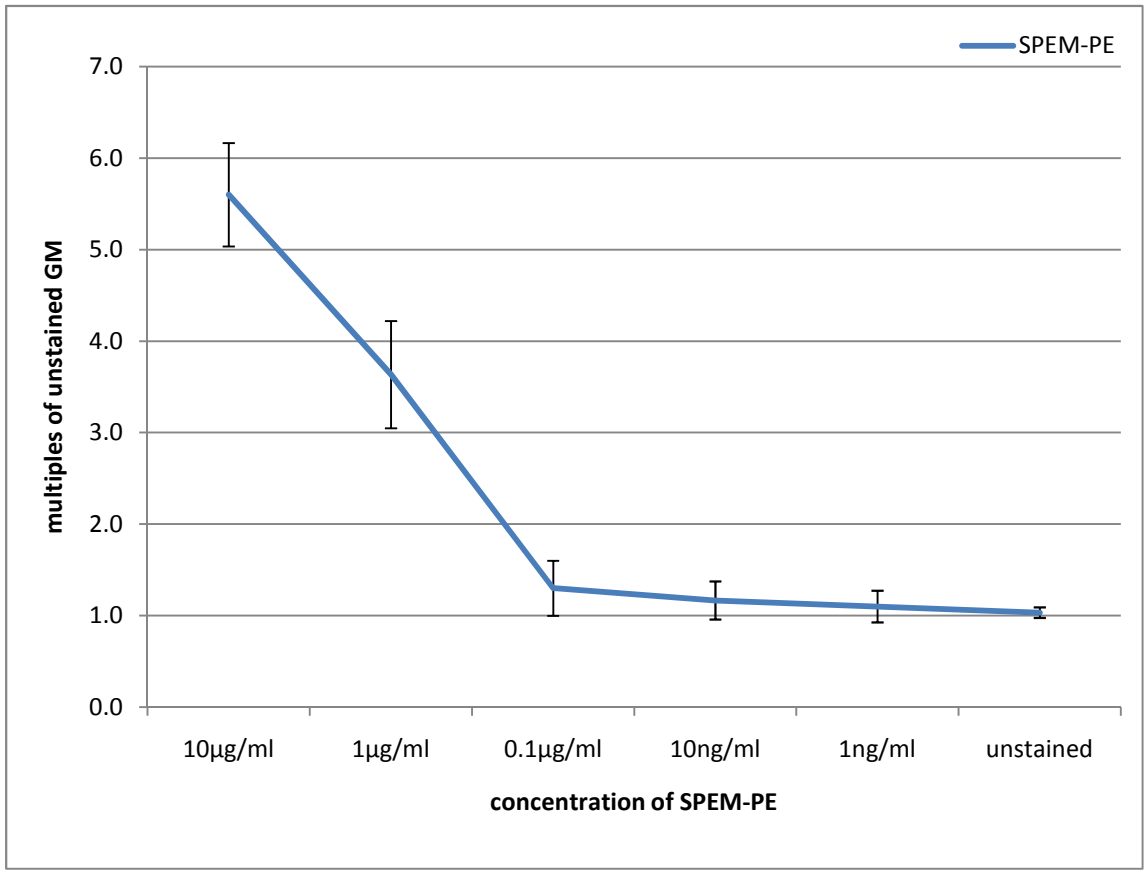


Figure 6.7 Titration curve for SPEM-PE

SPEM-PE demonstrated the greatest detectable binding. 1µg/ml toxin is sufficient to see a two fold increase in geometric mean compared to unstained cells. The P value at this concentration is 0.002 Points shown are a mean of triplicate readings from three separate experiments using the same batch of WT51 cells +/- 1 standard deviation. Staining was carried out on ice for 40 minutes in the dark.



6.3.2c Competition with HLA class II antibodies

In order to determine the relative roles of alpha and beta chain binding in interactions of SPEC, SPEL and SPEM the ability of antibodies targeting the DR α chain (L243) and pan class II β chain (TDR31.1) to inhibit superantigen binding to WT51 B cells was studied.

SPEC has been demonstrated previously to bind HLA class II via two zinc dependent β chain binding sites and no α chain binding site [24]. As TDR31.1 is a pan class II β chain antibody, if its epitope overlaps with the β chain SAg binding site, it would be expected that the antibody may block binding of SPEC to HLA class II. In keeping with this, prior incubation of WT51 cells with TDR31.1 resulted in dose dependent inhibition of superantigen binding (Figures 6.8 and 6.9). Even using a concentration of 10μg/ml antibody binding was not completely blocked. This concentration reduced binding of SPEC by 59.3%. Interestingly the same concentration of L243, an α chain antibody, also reduced binding of SPEC-PE by 24.1%.

The antibody competition assay was then carried out in triplicate using SPEL-PE and SPEM-PE using a range of concentrations of both antibodies. This aimed to determine the mechanism of SPEL and SPEM binding to HLA class II. In the presence of antibody to the HLA class II β chain binding of SPEL-PE was reduced by 23.1% and this was not statistically significant (P=0.07). No reduction of binding with L243 was observed (Figure 6.10).

10μg/ml of both TDR31.1 and L243 reduced binding of SPEM-PE by 41.9% and 45.3% respectively (P = 0.04 and 0.006 respectively). Dosage dependent inhibition was also observed for both antibodies (Figure 6.11). This may indicate that SPEM utilises both the α and β HLA class II binding sites. Complete inhibition of binding of both SPEC and SPEM was not observed.

Figure 6.8 Competition assay between SPEC-PE and HLA class II β chain antibody

Results shown are from a single experiment using the same batch of WT51 cells. Cells were stained on ice for 40 minutes in the dark. Geometric mean of SPEC-PE alone is 2.4 times larger than for SPEC-PE + TDR31.1 at 10µg/ml. Decreasing the concentration of antibody increases SPEC-PE binding to HLA class II β chain. At 0.1µg/ml antibody geometric mean is almost equal to SPEC-PE alone.

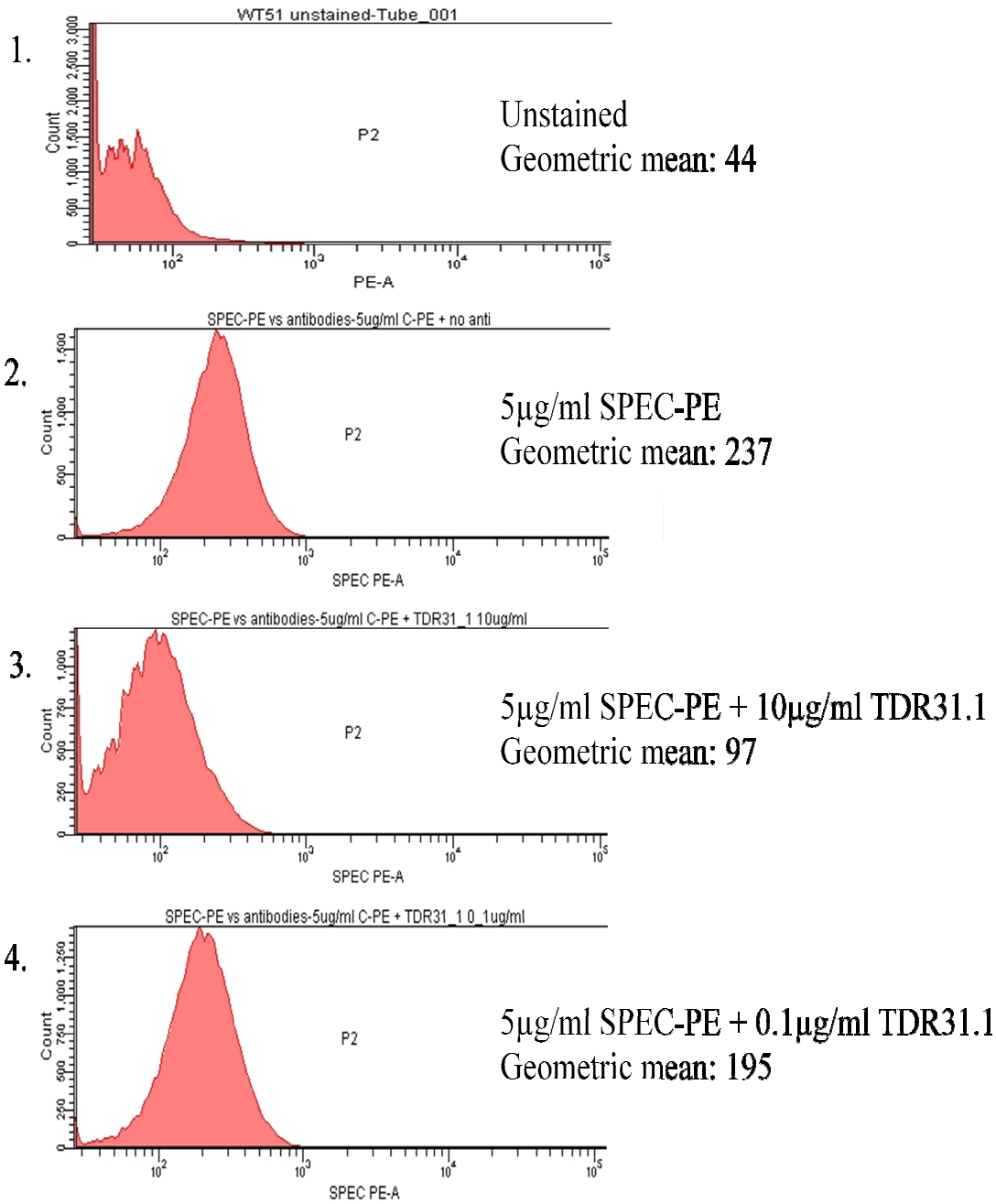


Figure 6.9 Inhibition of SPEC binding to WT51 B cells using antibodies to DR α chain (L243) or HLA class II β chain (TDR31.1)

TDR31.1 reduces SPEC binding by 59.3% at the highest concentration of antibody used. L243 reduces binding by 24.1%. No error bars are shown as a single experiment was conducted under these conditions. Cells were stained on ice for 40 minutes.

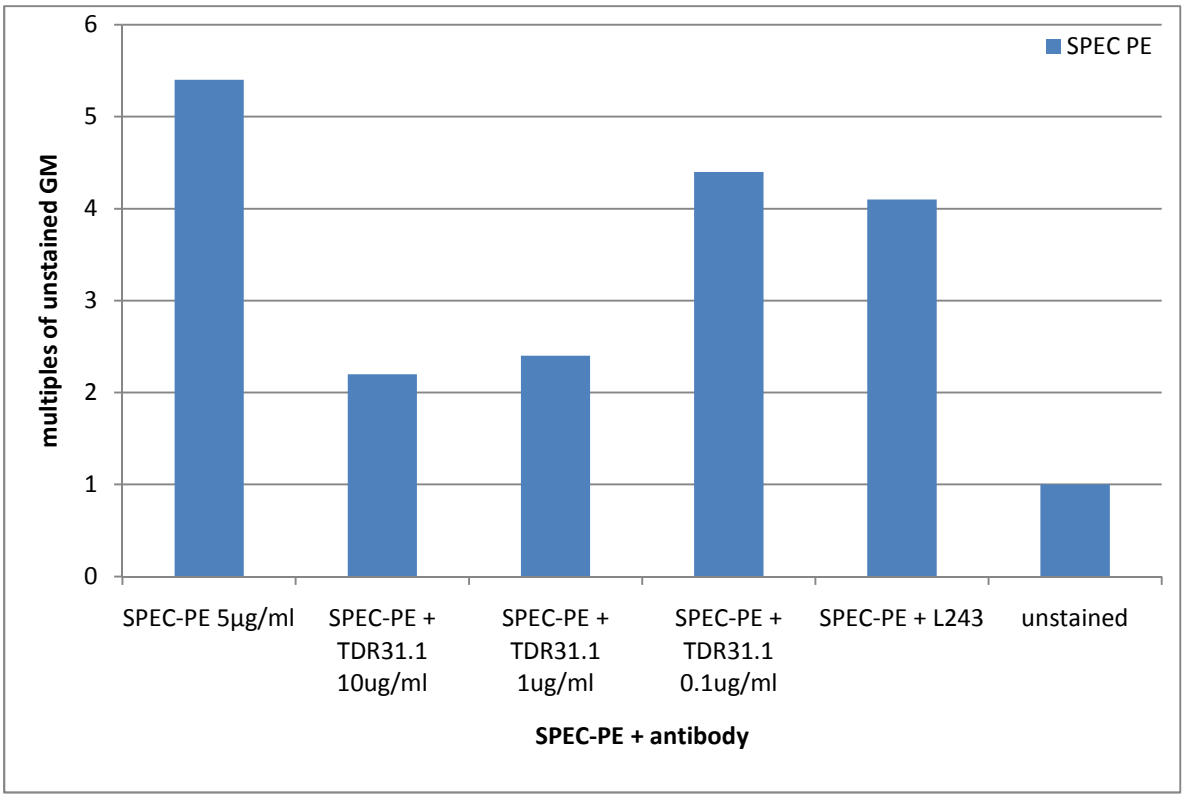


Figure 6.10 Inhibition of SPEL binding to WT51 B cells using antibodies to DR α chain (L243) or HLA class II β chain (TDR31.1)

Addition of TDR31.1 reduces binding of SPEL by 23.1% and this is not statistically significant (P=0.07). L243 does not inhibit binding of SPEL-PE to HLA class II. Points shown are a mean of triplicate readings from three separate experiments using the same batch of WT51 cells +/- 1 standard deviation. Cells were stained for 40 minutes on ice in the dark.

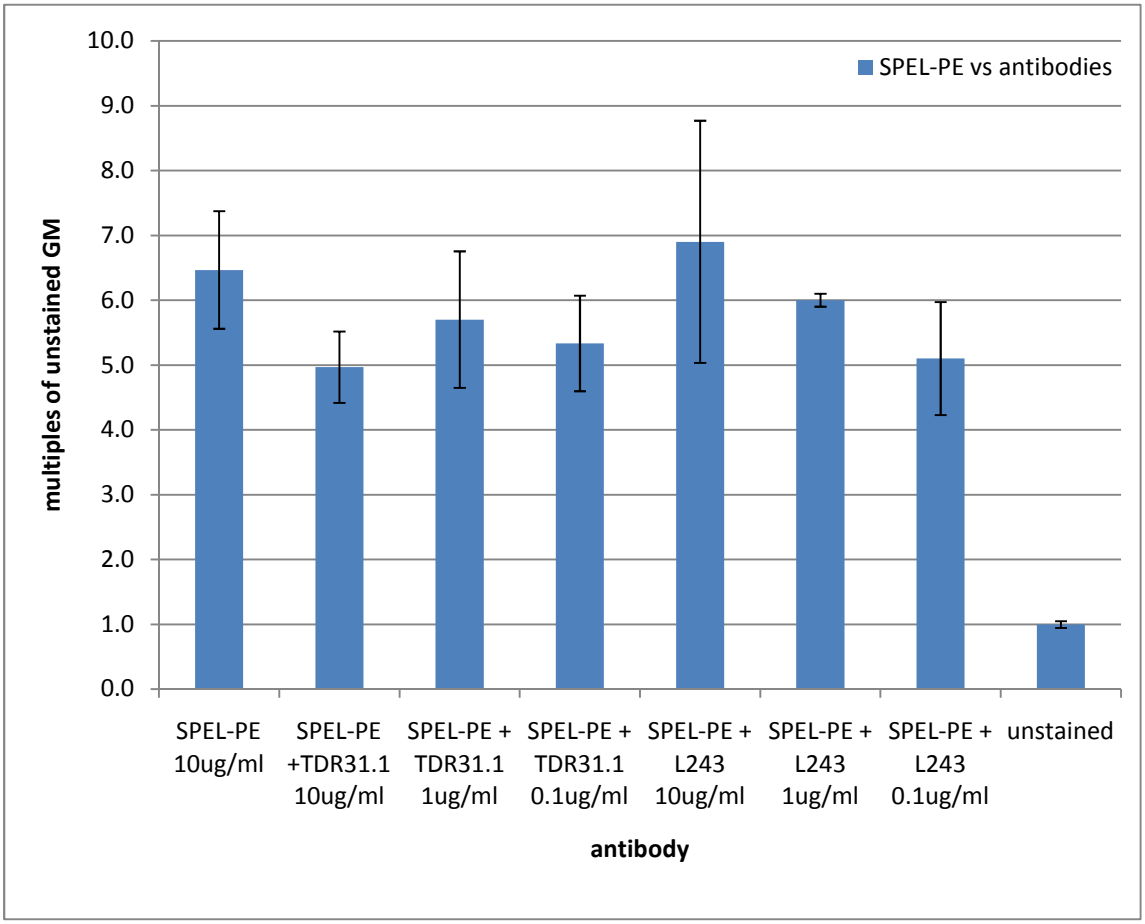
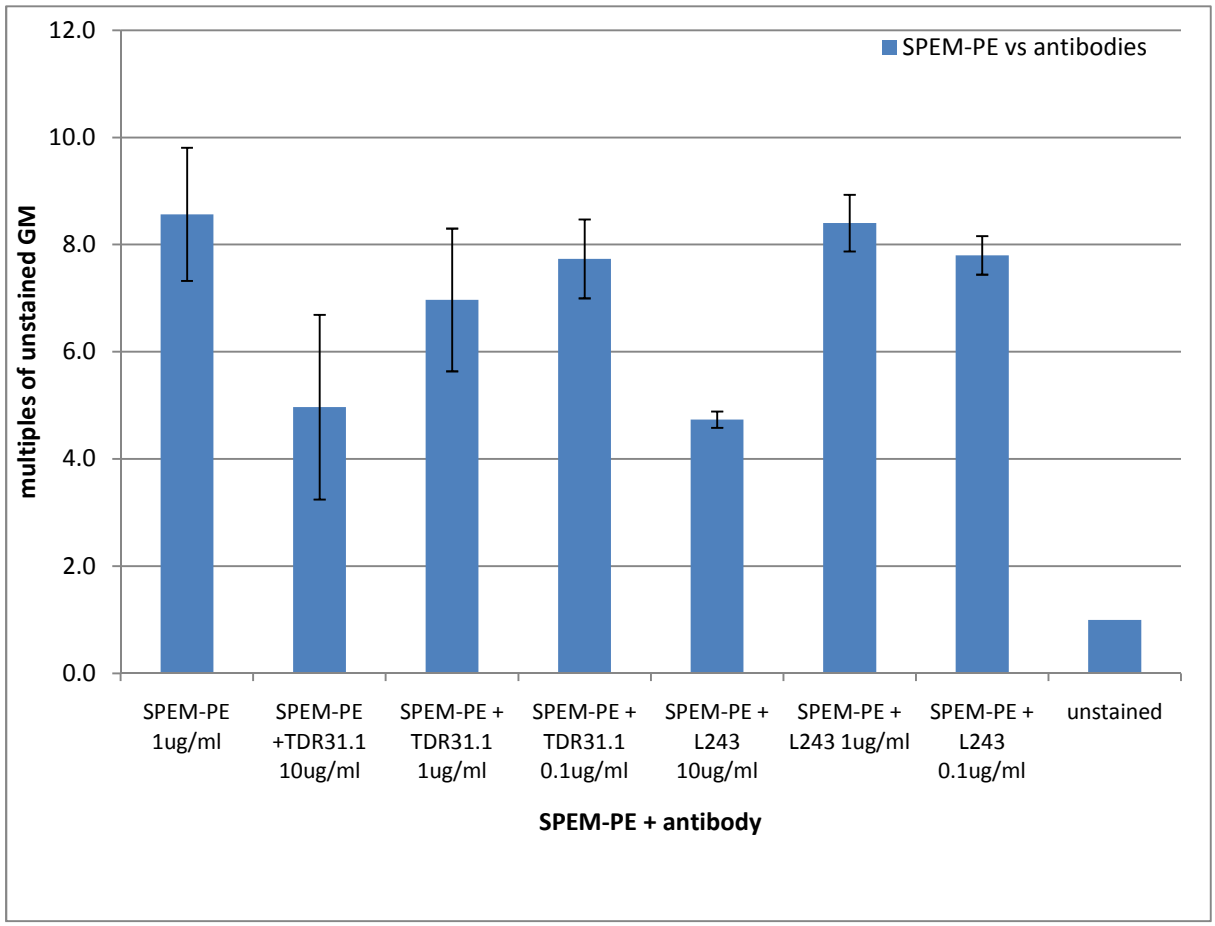


Figure 6.11 Inhibition of SPEM binding to WT51 B cells using antibodies to DR α chain (L243) or HLA class II β chain (TDR31.1)

Addition of TDR31.1 and L243 blocks binding of SPEM-PE to HLA class II in a dosage dependent manner. 10µg/ml TDR31.1 reduces binding of SPEM by 41.9% (P=0.04) and 10µg/ml L243 by 45.3% (P=0.006). Points shown are a mean of triplicate readings from three separate experiments using the same batch of WT51 cells +/- 1 standard deviation. Cells were stained for 40 minutes on ice in the dark.



6.3.3 Variability in Vβ specific responses to SPEM

Having demonstrated inhibition of binding of SPEM to WT51 B cells using anti HLA class II antibodies, further work was conducted to investigate the variability in T cell Vβ responses to SPEM between individuals described at the end of Chapter 3 (Figure 3.10a-d). Differences observed in superantigen responses have been described previously for other superantigens and attributed to HLA class II polymorphisms [66, 67, 203]. This formulated the hypothesis that HLA class II polymorphisms may contribute to variability in SPEM responses. In addition, previous evidence has suggested that SPEM utilises the HLA-DR β chain in its binding [58]. Work described in Chapters 5 and 6 of this thesis have also demonstrated the zinc dependent binding of SPEM to HLA class II, in particular binding to HLA-DR in Chapter 5, further indicating HLA class II β chain involvement in SPEM binding. Based on this, donors were HLA typed and grouped based on their *DRB1* alleles. *DQA1* and *DQB1* alleles were not used to group donors. It is possible these alleles may be involved in the binding of SPEM; however evidence suggests DR β binding is more important.

6.3.3a Influence of HLA class II on responses to SPEM

5 donors were grouped based either on identical homozygosity (Group 1) or on similarities between important superantigen binding sites on their individual *DRB1* alleles (Group 2). In summary groups were as follows:

Group 1:

LLEM 0 **Donor A** HLA-DR serotype 4, 4 (*DRB1*04* homozygous)

LLEM 4 **Donor B** HLA-DR serotype 4, 4 (*DRB1*04* homozygous)

Group 2:

LLEM 1 **Donor C** HLA-DR serotype 5, 6 (*DRB1*1101, 1301*)

LLEM 5 **Donor D** HLA-DR serotype 5, 7 (*DRB1*1101, 0701*)

LLEM 6 **Donor E** HLA-DR serotype 7, 7 (*DRB1*0701* homozygous)

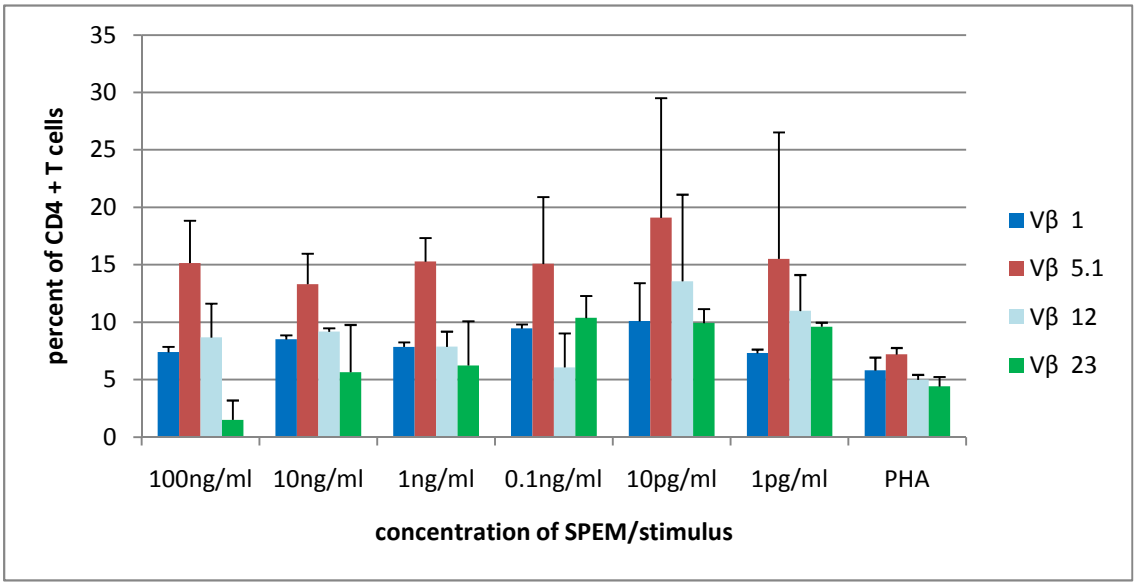
PBMCs from donors were stimulated with a range of concentrations of SPEM and Vβ signatures assessed by flow cytometry. Experiments were repeated a total of three times with each donor and averaged data plotted for each group. Vβ specific changes in T cell repertoire following SPEM stimulation are shown for these donors in Figure 6.12a and b. Interestingly, in contrast to the 3 donors used in Chapter 3 (Figure 3.10a-d) where SPEM primarily expanded Vβ1, in these experiments SPEM to targetted mainly Vβ5.1 in both groups. The proportion of Vβ5.1 peaks at 10pg/ml in both groups. Vβ5.1 also expands in a dosage dependent manner,

increasing as concentration of toxin decreases. Vβ23 shows a similar trend, although the magnitude of Vβ23 specific proliferation was slightly greater in Group 2. Very little response is seen in Vβ1 in either group; however Vβ12 seems to expand in a dosage dependent manner, peaking at 10pg/ml for Group 1 and 1pg/ml for Group 2. This is in contrast to Donors 1-3 in chapter 3 above. Vβ12 has not previously been documented to respond to SPEM [58, 164] so this was unexpected, however it may indicate that Vβ12 could expand in some individuals in response to SPEM. In each case between groups the proportions of individual Vβs do not add up to 100%. The remaining percentage may be a sum of other non-responding Vβs. Alternatively other Vβs not studied here may expand.

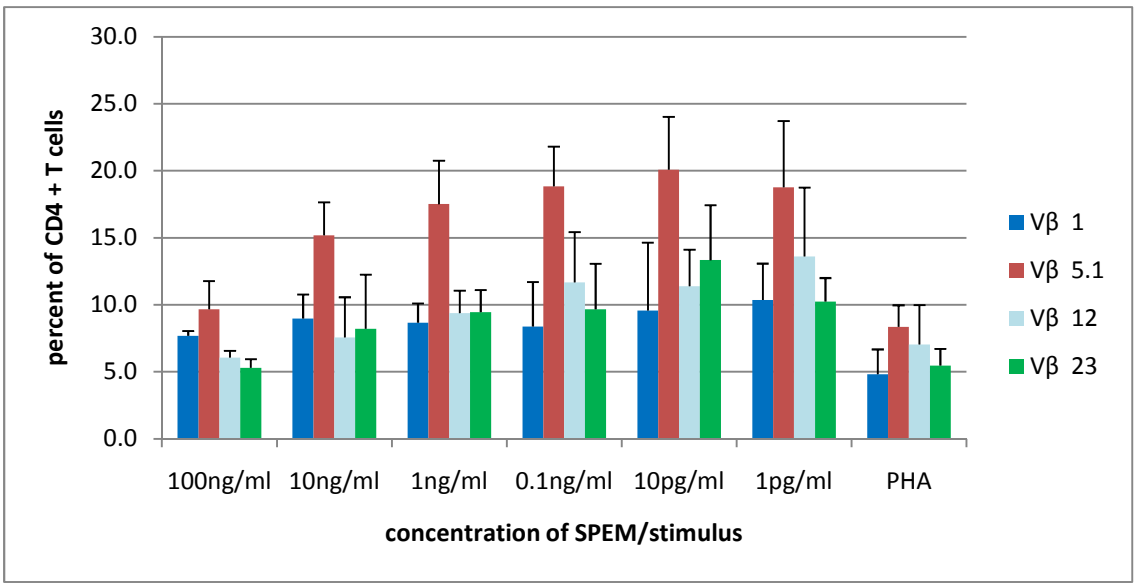
Figure 6.12 Vβ signatures in response to SPEM between groups of HLA-DR β chain similar donors

Vβ5.1 and to a lesser extent Vβ23 expand, increasing as concentration decreases in both groups. Little expansion is seen in Vβ1 however Vβ12 does appear to expand in a similar trend to Vβ23. Points shown in each case are the mean of three separate experiments for each donor conducted on three separate days. PBMCs from each donor were stimulated with SPEM for 48 hours before staining and flow cytometric analysis.

a) Group 1 average – Donors A + B (HLA serotypes 4, 4 and 4, 4 respectively) Points shown are the means of triplicate readings from two donors plus 1 standard deviation



b) Group 2 average – Donors C, D and E (HLA serotypes 5, 6; 5, 7 and 7, 7 respectively) Points shown are the means of triplicate readings in three donors plus 1 standard deviation



Although obvious trends in Vβ expansion were apparent in averaged groups data and there were no key differences between the groups themselves, these trends did contrast with earlier findings described in Chapter 3, for example Vβ5.1 seemed to be the primary target here compared to Vβ1 in Chapter 3 whilst Vβ12 appeared to expand here in contrast to three donors used in Chapter 3.

6.3.3b Novel differences in SPEM responses between individuals

Another key observation made during this work was that although trends across and between groups were evident; there was clearly a great deal of variation, both between individuals and between experiments in the same donor. Therefore a decision was made to analyse the individual results more closely.

Results were plotted for each individual experiment for each donor before averaging to observe general trends (Figures 6.13-6.17). Overall (panel d in each case) it could be seen that Vβ5.1 was the primary Vβ expanded, however the pattern of Vβ5.1 expansion was markedly different between donors. Donors A, D and E showed very similar patterns of Vβ5.1 expansion in a dosage dependent manner, peaking at 10pg/ml in each case; however the magnitude of Vβ5.1 specific proliferation varied between 18-27% between these donors. These donors also showed similar patterns of Vβ23 and 12 expansion to a lesser extent. A general overall trend for these three donors was that proportions of all Vβs expanding increased with decreasing concentration of toxin. This is in contrast to findings described in Chapter 3 for both SPEL and SPEM where the Vβ signature spread with stimulus strength. The overall magnitude of Vβ specific proliferation was lower in Donor E than Donors A and D despite overall trends being very similar. An interesting observation was that Donors B and C showed very little Vβ specific expansion in any Vβ, and the proportion of any Vβ was generally lower than in other donors, not reaching above 20% with any Vβ studied. It is also noteworthy that similar trends in Vβ expansion to SPEM were observed between donors, but these donors did not necessarily share any common *DRB1* alleles (Donors A + D or B + C for example).

6.3.3c Novel differences in SPEM responses between days

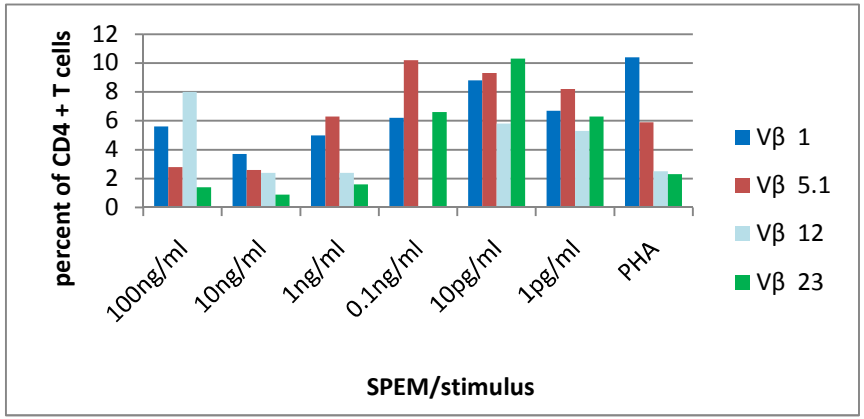
In addition to the variation in Vβ responses to SPEM described between donors, when observing individual experimental data for each donor, it was apparent that responses also seemed to vary considerably between days in the same donor (Figures 6.13-6.17, panels' a-c in each case). As with the overall trend the Vβ specific response in Donor B (Figure 6.14) was not clear between experiments and the proportion of Vβ specific T cells was generally lower than in other donors. A similar pattern was seen with Donor C in two out of three experiments (figure 6.15a and c), although in the second experiment a dosage dependent expansion in Vβ5.1 was seen (Figure 6.15b). Trends were apparent between experiments in the same donor, for example

Donor A showed an expansion in Vβ5.1 in experiments 2 and 3 (Figure 6.13b and c), but the proportion of Vβ5.1 positive T cells peaked at concentrations which were 1 log different (1pg/ml and 10pg/ml respectively). This was also observed with Donor D and E (Figure 6.16 and 6.17). These variations together with others which can be observed in Figures 6.13-6.17 appear to indicate that Vβ responses to SPEM differ not only between donors of the same and differing HLA type, but also between days in the same individual. This suggests again that HLA class II polymorphisms cannot be the only contributing factor in varying responses to SPEM and therefore that HLA class II polymorphisms could not be used to predict outcome or severity of disease with SPEM.

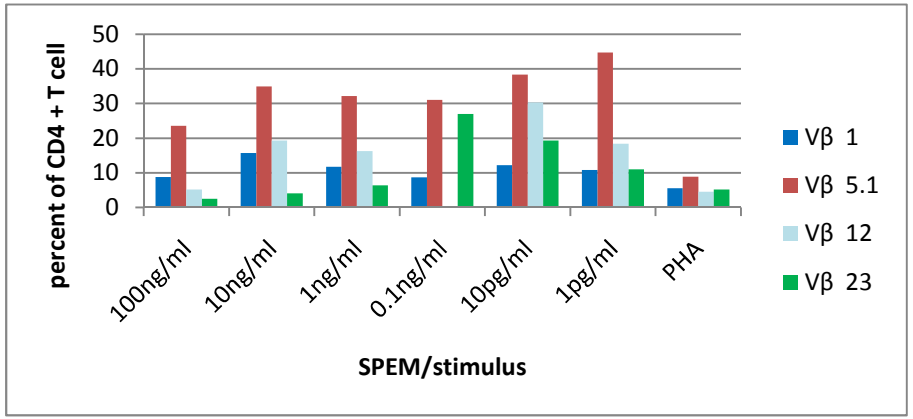
Figure 6.13 Variability in responses to SPEM in Donor A

Points shown in panels a-c are for three individual experiments for donor A whilst panel d is the average of the three experiments +/- 1SD. PBMCs were stimulated with SPEM for 48 hours in each case before staining and flow cytometric analysis. a, b, c) Repetitions 1, 2 and 3, d) average. Showing differing patterns of Vβ response between experiments although Vβ5.1 remains the primary Vβ expanded. Overall (d) Donor A shows an expansion in Vβ5.1 in a dosage dependent manner. Blank bars in repetition 1 and 2 (a and b) indicate no data for those points) Inconsistent involvement of other Vβs is observed.

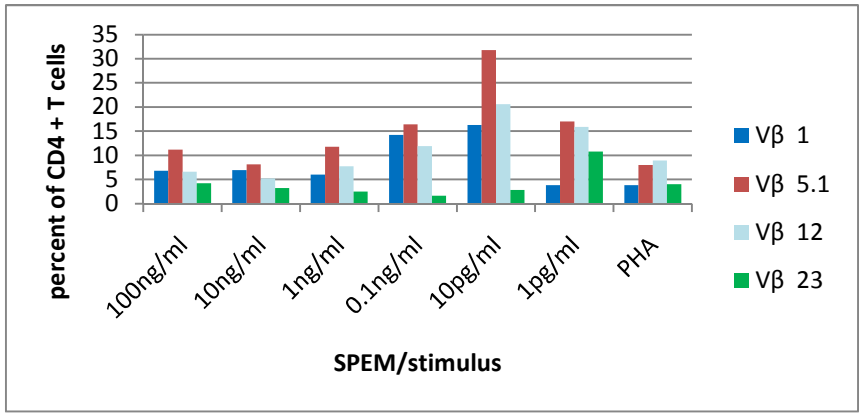
a)



b)



c)



d)

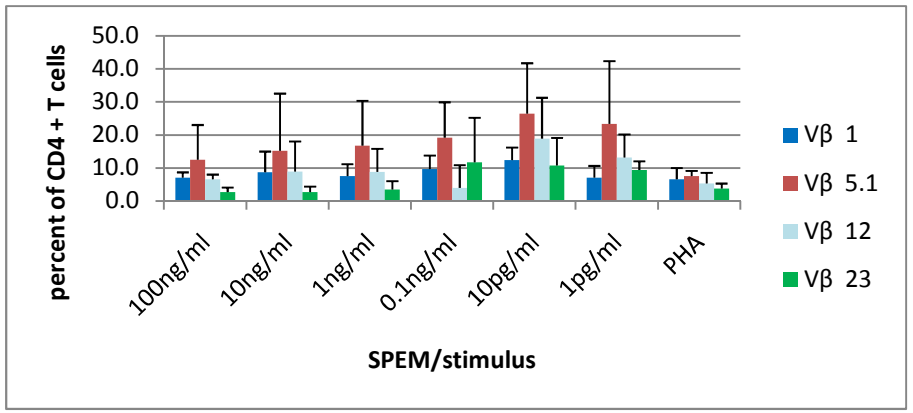
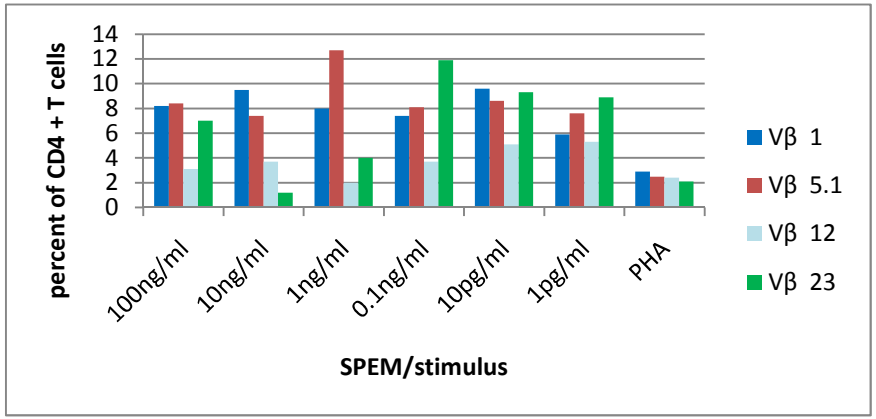


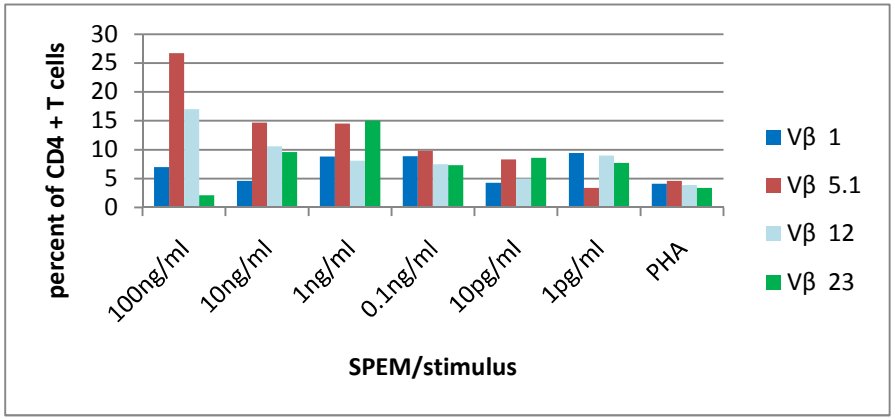
Figure 6.14 Variability in responses to SPEM in Donor B

Points shown in panels a-c are for three individual experiments for donor B whilst panel d is the average of the three experiments +/- 1SD. PBMCs were stimulated with SPEM for 48 hours in each case before staining and flow cytometric analysis. a, b, c) Repetitions 1, 2 and 3, d) average. Showing very varying patterns of Vβ response between experiments with no consistency in Vβs expanded. No dosage dependent expansion was observed in any experiment. Overall (d) Donor A shows no trend in expansion of any Vβ and generally displays a poor Vβ specific response.

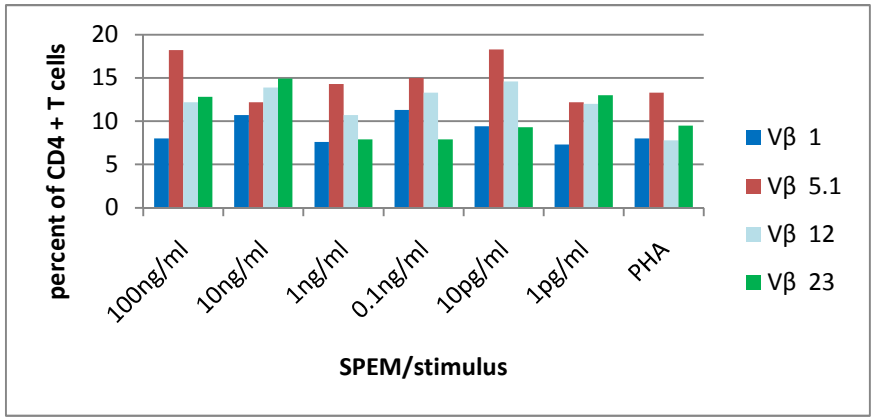
a)



b)



c)



d)

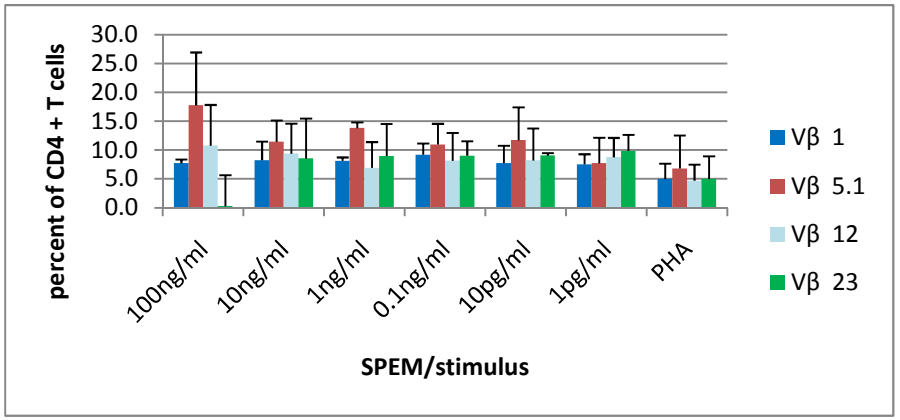
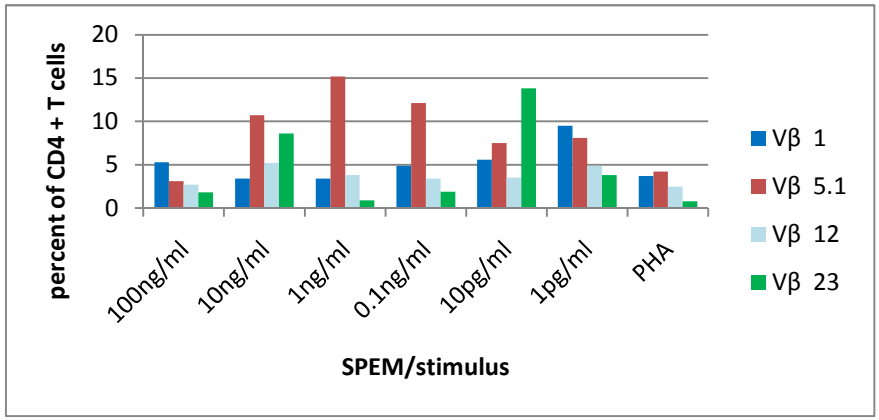


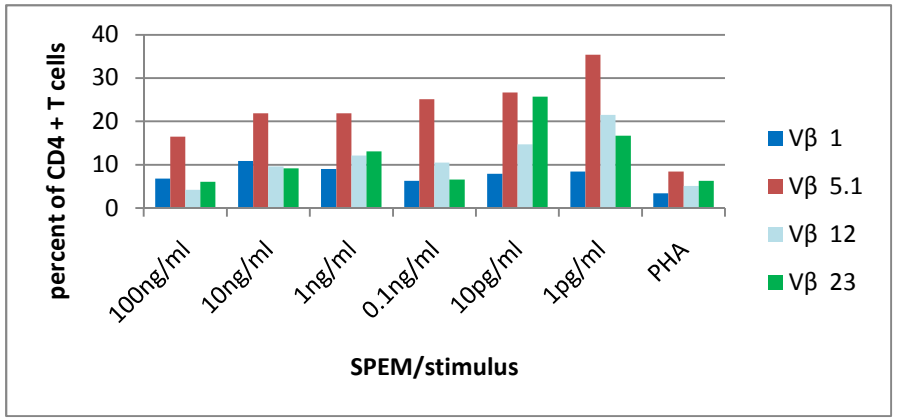
Figure 6.15 Variability in responses to SPEM in Donor C

Points shown in panels a-c are for three individual experiments for donor C whilst panel d is the average of the three experiments +/- 1SD. PBMCs were stimulated with SPEM for 48 hours in each case before staining and flow cytometric analysis. a, b, c) Repetitions 1, 2 and 3, d) average. Showing differing patterns of Vβ response between experiments. In repetition 1 (a) Vβ5.1 expands with increasing concentration, in contrast to repetition 2 (b). Vβ23 expands with decreasing concentration in repetitions 1 and 2 but with increasing concentration in repetition 3. Repetition 3 shows little specific Vβ response (c). Overall (d) Vβ5.1 is the primary Vβ expanded, but overall pattern is similar to Donor B.

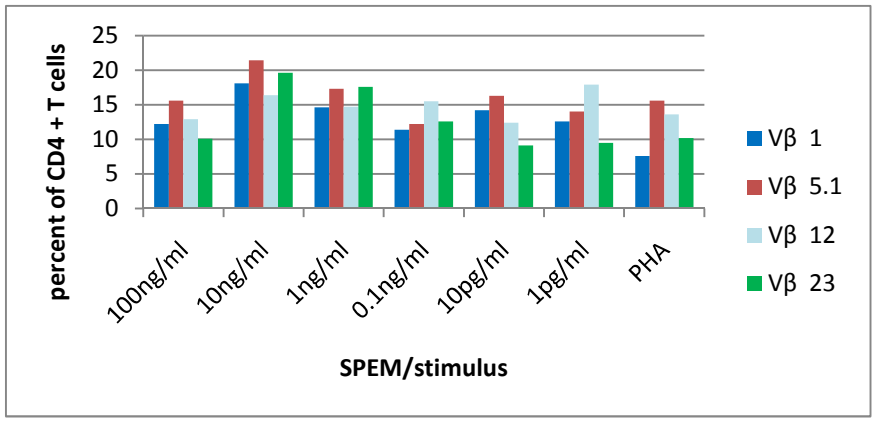
a)



b)



c)



d)

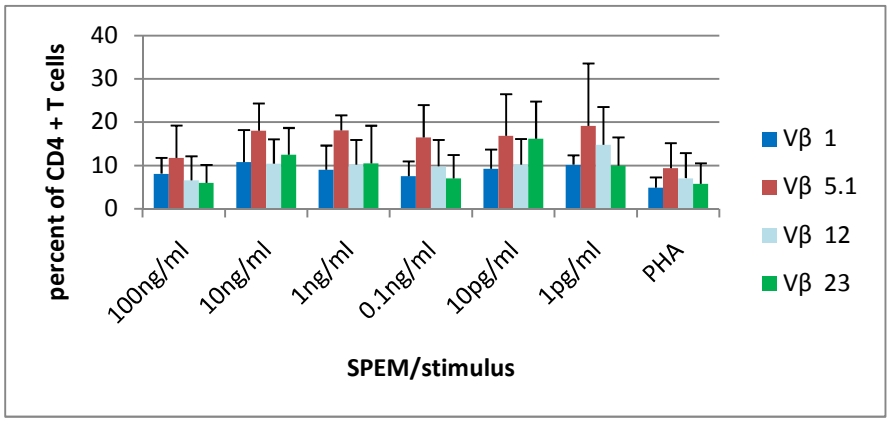
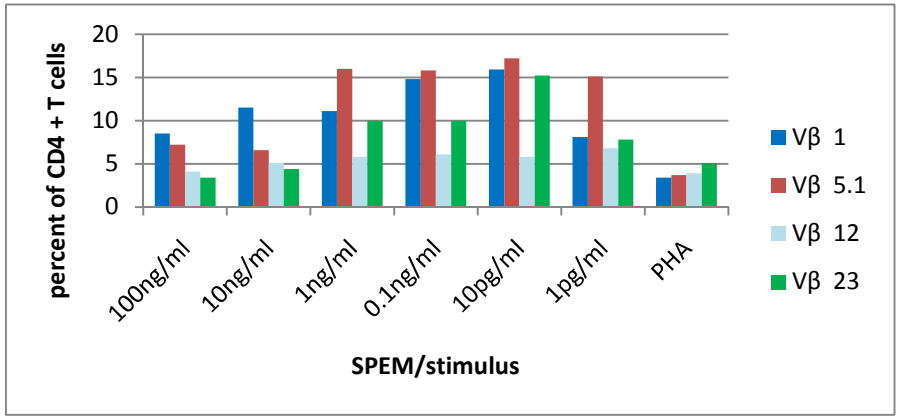


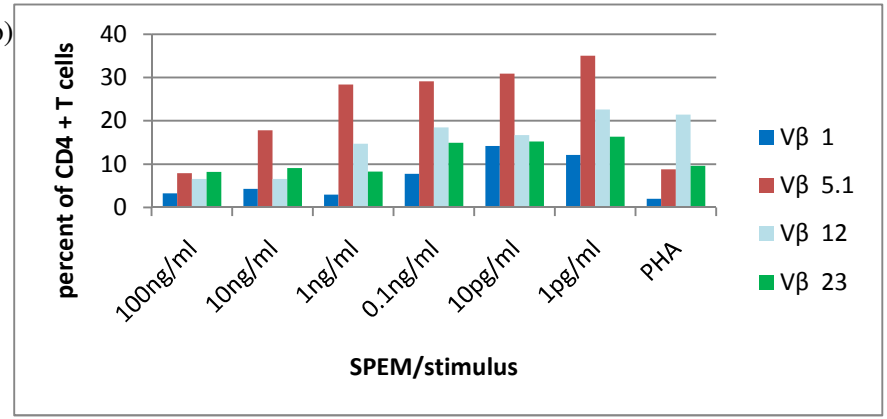
Figure 6.16 Variability in responses to SPEM in Donor D

Points shown in panels a-c are for three individual experiments for donor D whilst panel d is the average of the three experiments +/- 1SD. PBMCs were stimulated with SPEM for 48 hours in each case before staining and flow cytometric analysis. a, b, c) Repetitions 1, 2 and 3, d) average. Showing Vβ5.1 expansion with decreasing concentration in all repetitions. Vβ1 expands in the same pattern in repetitions 1 and 2 (a and b). Vβ23 involvement is less consistent between experiments, as is Vβ12 involvement. Overall (d) Vβ5.1 is the primary Vβ expanded, with smaller responses seen in Vβ1 and 23.

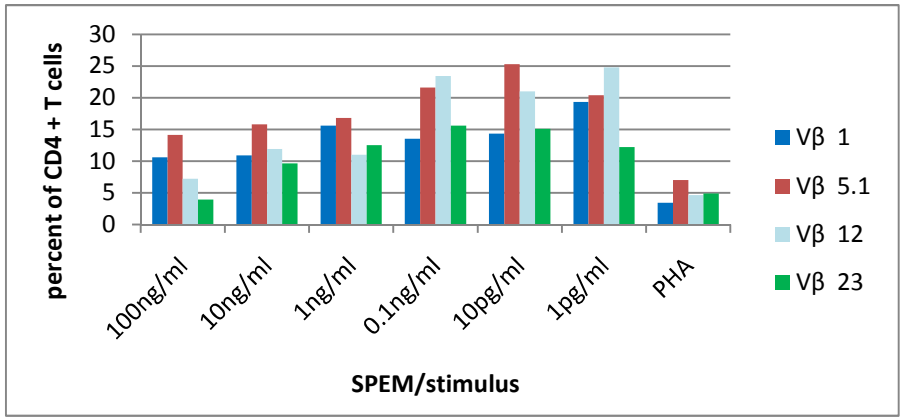
a)



b)



c)



d)

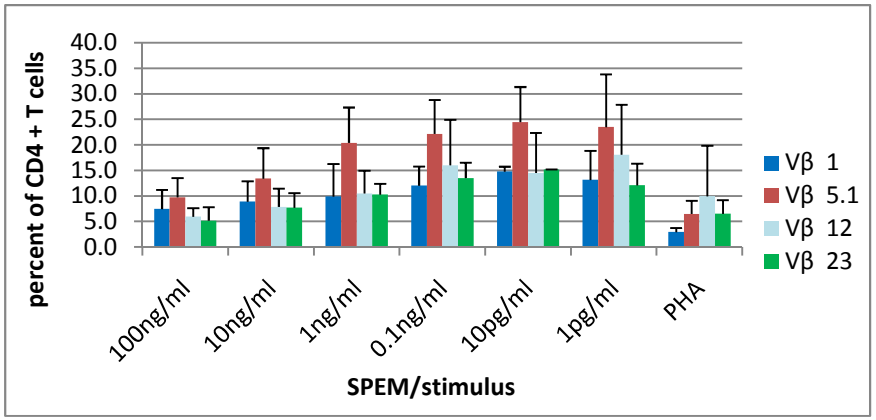
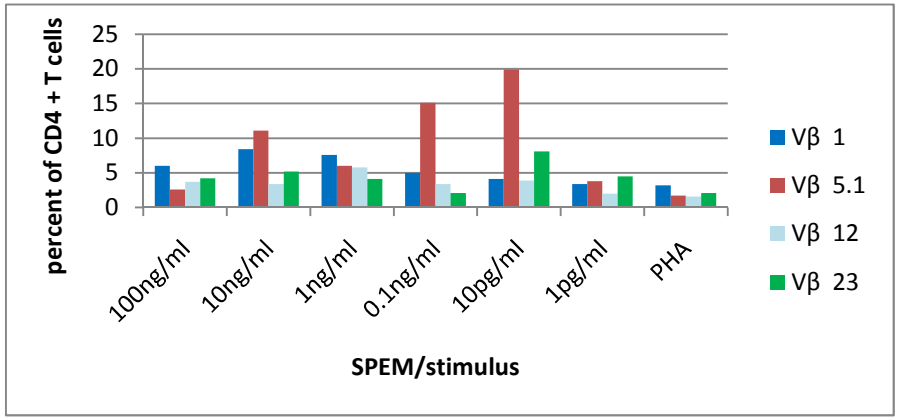


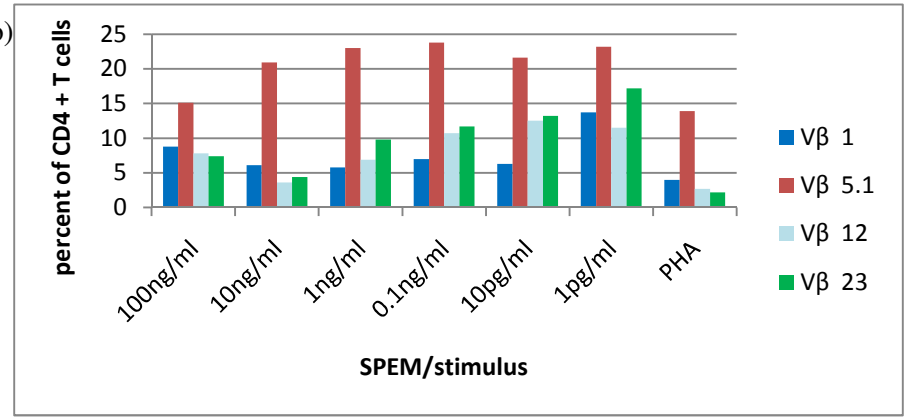
Figure 6.17 Variability in responses to SPEM in Donor E

Points shown in panels a-c are for three individual experiments for donor E whilst panel d is the average of the three experiments +/- 1SD. PBMCs were stimulated with SPEM for 48 hours in each case before staining and flow cytometric analysis. a, b, c) Repetitions 1, 2 and 3, d) average. Expansion of Vβ5.1 is seen in all repetitions and is most dosage dependent in repetition 3 (c). Differing dosage dependent responses to Vβ23 are seen in repetitions 2 and 3 (b and c), with no clear involvement in repetition 1. Vβs 1 and 12 display inconsistent involvement. Overall (d) Vβ5.1 is the primary Vβ expanded in a dose dependent trend similar to Donors a and d.

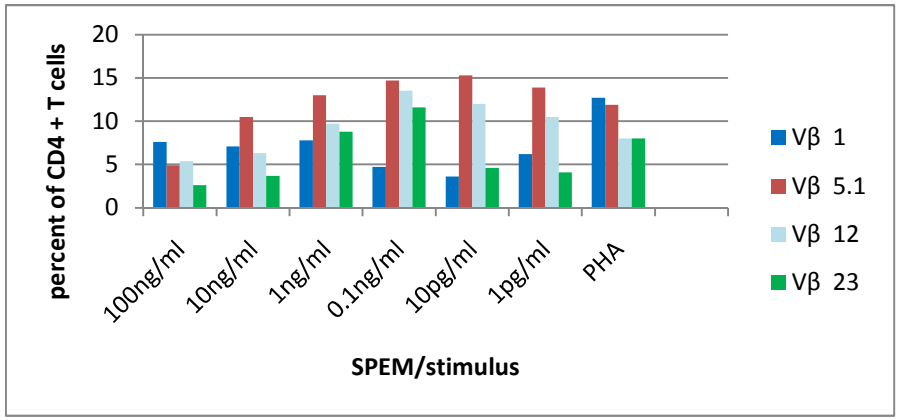
a)



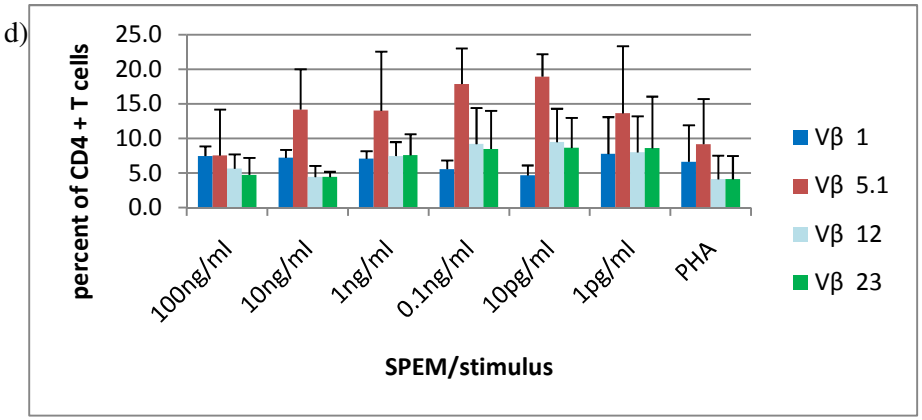
b)



c)



d)



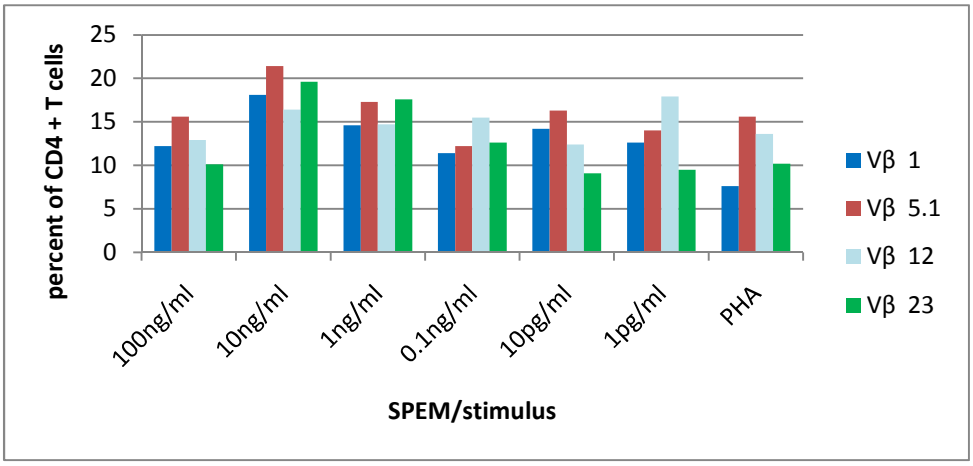
Finally, in order to deal with the possibility that the large intra-individual variation in responses to SPEM represented experimental variation, two observations were made in the same individual on the same day. This was in contrast to previous experiments where all donors were studied concurrently on the same day under the same tissue culture conditions and staining procedures, but repeats were conducted on separate days. This aimed to show that variability between responses was not due to individual to individual experimental conditions such as differences between tissue culture, staining or flow cytometry. Identical gates were used for the duplicate experiments and cells were incubated with toxin in the same position in the incubator, as well as in the same wells on separate plates. In this case, repeats on the same day using the same donors blood (Donor C) showed no discernable trends in Vβ expansion in either case (Figures 6.18a and b), with the exception of Vβ23 which expands with increasing concentration in both cases. Some variability between experiments was observed, but not to the same extent as earlier experiments between the same donor and other donors. Standard deviation shows us (Figure 6.18c) that with the exception of one or two points, Vβ proportions were very similar between the two experiments.

Figure 6.18 Comparison of Vβ responses to SPEM in one donor on the same day

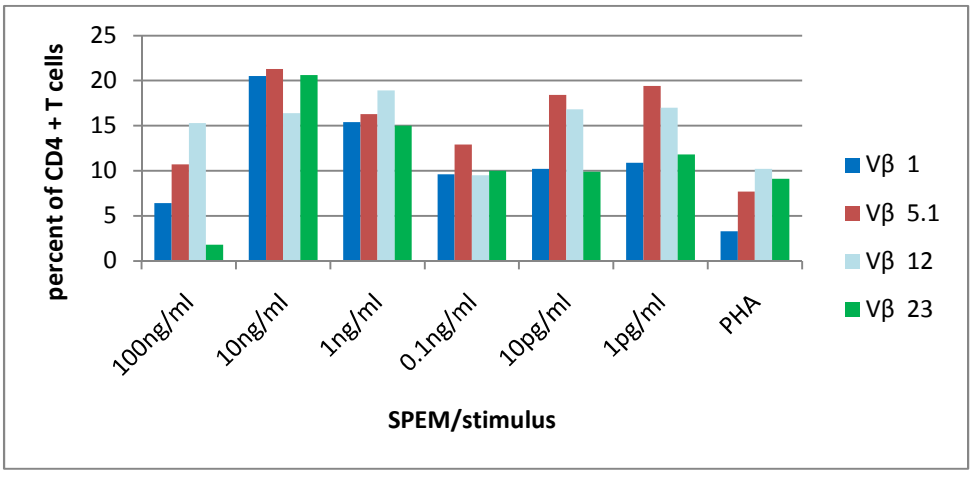
Points shown in panels a+b are for two individual experiments for donor C conducted on the same day whilst panel c is the average of the three experiments +/- 1SD. PBMCs were stimulated with SPEM for 48 hours in each case before staining and flow cytometric analysis.

a, b) repetition 1, 2 c) average. Showing an expansion in Vβ23 with increasing concentration of toxin but no discernable trends in other Vβs in both repeats. Importantly, average data indicates a far lower degree of variability than observed between donors and experiments at other times. Points shown in panel c are the mean of duplicate readings plus 1 standard deviation.

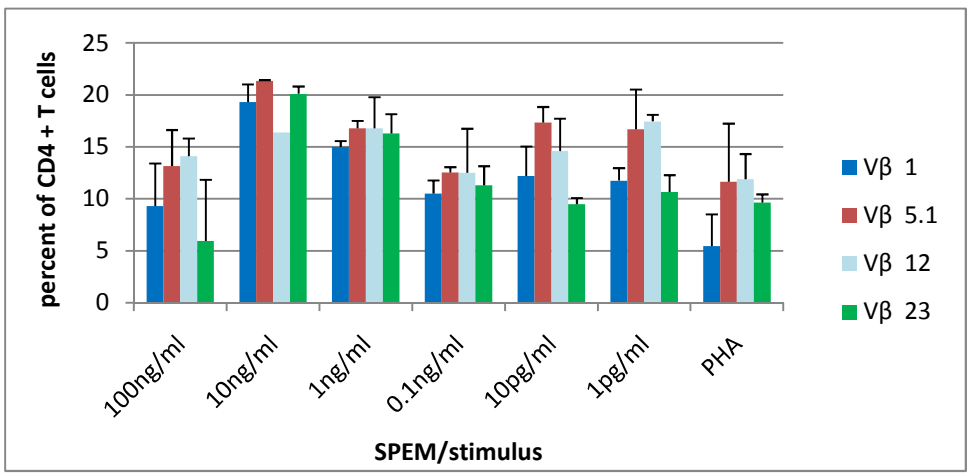
a)



b)



c)



6.4 Discussion

6.4.1 Verification of superantigenicity of PE conjugated SAgs

Initial work in this chapter aimed to demonstrate binding of superantigens to HLA class II using an HLA homozygous B cell line WT51 and PE conjugated SAg. Figure 6.1 shows that conjugation of SAg with PE did not affect superantigens ability to produce Vβ specific responses. Very low Vβ specific proliferation was observed with SPEC-PE compared to results shown in Figure 3.6 in Chapter 3; although a Vβ specific response was still seen with Vβ12. It is possible that PE conjugation of SPEC partially blocked HLA class II binding sites on the toxins, perhaps accounting for lower proportions of Vβ specific T cells observed here. SPEL caused expansion in Vβs 1, 5.1 and 23 to a lesser extent whilst SPEM expanded the same three Vβs with differing proportions to SPEL, similar to results observed in Chapter 3 (Figures 3.7-3.9).

Expression of HLA class II on WT51 cells was successfully verified by flow cytometry and anti HLA class II antibodies labelled with FITC. All PE conjugated SAgs were used successfully in initial flow cytometric binding assays (Figure 6.4). Interestingly an initial experiment using FACS buffer with no additional zinc resulted in no detectable binding of any SAg-PE; however the addition of zinc resulted in very easily detectable level of binding of each toxin. This agrees with published evidence that SPEC binds HLA class II in a zinc dependent manner [24, 32] and reinforces the hypothesis that SPEL and M utilise the HLA class II zinc dependent β chain binding site [7, 26]. SPEC and SPEM demonstrated a decreasing level of fluorescence and therefore inferred binding with decreasing concentration, whilst SPEL demonstrated a lower level of fluorescence overall, indicating either that SPEL was not as saturated with PE as SPEC or SPEM, or that SPEL binds HLA class II with lower affinity than either SPEC or SPEM. This cannot be determined further without use of SPR or other similar techniques.

Competition for binding of HLA class II between SAg-PE and anti class II antibodies was used to determine which sites SPEC, L and M bind on HLA class II. SPEC has been previously demonstrated to bind HLA-DR β chain with high affinity [7, 24, 69] and has been hypothesised to contain two HLA class II β chain binding sites and no α chain binding site [24]. Figures 6.8 and 6.9 reinforce this, showing a dosage dependent blocking of SPEC binding with the use of TDR31.1 (a class II β antibody) compared with L243 (anti HLA-DR α chain). Interestingly some reduction of binding was also observed with L243. It is possible that this reduction in binding is a result of stearic hindrance due to the large size of immunoglobulin perhaps interfering with the SAg binding sites on HLA class II. However in view of recent evidence that SPEC may in fact possess a low affinity 'interface' for binding HLA class II α chain [56], this result could also indicate α chain involvement in SPEC recognition to a lesser extent than β chain involvement. Further work using a concentration range of L243 antibody could elucidate

this further. Surface plasmon resonance (SPR) analysis using HLA class II α chain to bind SPEC could also be used as a different approach.

Addition of TDR31.1 reduced binding of SPEL by 23.1% and this was not statistically significant. L243 did not inhibit binding of SPEL-PE (Figure 6.10). This may indicate that SPEL utilises the HLA-DQ α chain in its binding, although zinc dependent binding of SPEL has been demonstrated in this chapter (Figure 6.4). Another possibility is that the mechanism of SPEL binding HLA class II β chain may be in a conformation which is not inhibited by TDR31.1.

Finally, SPEM binding to HLA class II was blocked in a dosage dependent manner by TDR31.1. L243 also blocked binding at the highest concentration however these data, as well as data described in Chapter 5 (Figure 5.4c) indicate that SPEM does in fact bind HLA class II β chain with zinc dependence. α chain binding may also play a role in the stimulation of T cells by SPEM. As SPEL and SPEM are grouped in the same subfamily of toxins as SPEC, it could be hypothesised that these toxins may also contain a low affinity 'interface' for the binding of HLA class II α chain.

Addition of antibody did not completely inhibit binding of SAg-PE to WT51 B cells with any toxin. This suggests that some binding detected was non-functional, i.e. SAgs were not just binding to HLA class II but also to other receptors on the cell surface. This could be investigated by determining the ability of these cells to present superantigen to purified T cells. B cells incubated with superantigen alone, and superantigen and antibody could be compared in their ability to induce Vβ specific T cell proliferation in purified T cells or T cell clones.

Various experiments could be conducted to further elucidate the binding mechanisms of SPEC, L and M. SPR could be used for example utilising recombinant HLA class II α and β chain immobilised on a chip to capture superantigens. Additionally, ELISA based assays similar to those described in Chapter 5 could be developed using HLA class II α and β chain to detect a range of immobilised superantigens. The use of anti-HLA class II antibodies to inhibit SAg binding could also be used in this approach, as well as investigated further in flow cytometric assays.

6.4.2 Influence of HLA type on the Vβ signature of SPEM

Following a demonstration of superantigenicity of SPEC, L and M and from observations of varying responses to SPEM between donors described in chapter 3, Vβ responses to SPEM were studied in a range of donors of grouped HLA type, in order to investigate the hypothesis that HLA class II polymorphisms have an effect on the magnitude and hierarchy of Vβ responses to SPEM, which has been previously demonstrated for other superantigens including SPEA, SPEC and SEA [66, 67]

Donors were grouped based on similarity between amino acids at key residues in the HLA class II β chain identified in crystallographic studies of superantigens in view of the hypothesised β chain binding of SPEM [26, 58] and the zinc dependent binding demonstrated for this superantigen in this chapter and Chapter 5. It was not possible to group donors based on HLA-DQ α or β chain similarity as allelic differences were too marked between donors. Although HLA-DQ differences have the potential to influence superantigen responses, linkage disequilibrium exists between many HLA-DR and HLA-DQ types, meaning certain DQ types are commonly associated with certain DR types [204]. therefore grouping donors based on their HLA-DR type should result in fairly similar HLA-DQ types between groups and thus should not cause a great variation in superantigen responses unaccounted for by DR differences.

SPEM is documented to expand Vβs 1, 5.1 and 23 [58]. Differences in Vβ response were initially studied between the two groups, which showed similar overall trends between groups. Both groups showed a dosage dependent response to Vβ5.1, with proportions of this Vβ increasing with decreasing concentration of toxin, peaking at 10pg/ml in both groups. This is in contrast to Donors 1-3 used in Chapter 3 who showed a primary expansion in Vβ1. A similar trend was seen in Vβ23 whilst proportions of Vβ1 remained fairly constant throughout. Interestingly Vβ12 also responded in a dosage dependent manner in both groups, which has not previously been reported to expand in response to SPEM (Figure 6.12). This suggests there may be similarities between Vβs 1, 5.1, 23 and 12 at sites important in SPEM binding that are not apparent by phylogeny alone (Figure 1.4 in Chapter 1). Investigation of Vβ expansion in response to SPEM using multiplex PCR could help to elucidate this further, as well as crystallographic studies of SPEM complexed with TcR Vβ.

The two groups used were very similar in the amino acids present at important SAg binding sites, only differing at residue 70 (glutamine in Group 1, aspartate in Group 2, which perhaps accounts for the very similar trends observed. However when the *DRB1* sequences are studied in wider detail than the specific amino acids used to group donors, there are differences in the sequences surrounding the conserved histidine residue at position 81. For example, at position 74, not currently reported to be an important site in SAg binding, *DRB1*0403* (Donor B) contains a glutamate (E) residue, whilst *DRB1*1101* (Donors C and D) and *DRB1*0701* (Donors D and E) contain alanine and glycine residues respectively at this position which differ considerably in charge and size from glutamate (further particulars of *DRB1* sequences can be found in appendices 1 and 3). This is interesting to note as although similar trends between groups were observed, standard deviation across averaged data indicated a very high level of variability between individuals, suggesting that although key amino acids may be identical between individuals, overall HLA class II structure may differ enough to cause variation in response between individuals.

When investigating the variability observed between individuals in more detail it was apparent that not only did variation in Vβ expansion exist between individuals, but also on a day to day basis between the individuals themselves. Several of the donors used showed marked differences in their Vβ responses to SPEM between experiments, and no consistent trend in this pattern was observed (Figures 6.13-17). Of particular note were instances where the proportion of the primary Vβ responding, in general Vβ5.1, peaking at concentrations 10 or even 100 fold different between experiments (Donor A, repetitions 2 and 3 for an example Figure 6.13a+b). In addition, some donors showed a completely different trend in the expansions of particular Vβs; for example Donor C showed an increasing proportion of Vβ5.1 with decreasing concentration of toxin in repetition 2 (Figure 6.18b) and the opposite trend in repetition 3 (Figure 6.16c). Donors D and E, who share an allele for *DRB1*0701* demonstrated very similar overall trends, indicating that this allele in particular may contribute to differences in Vβ response. Interestingly Donors B and C showed very few discernable trends in individual experiments. It is possible that for these individuals other Vβs not studied here may expand, suggesting that SPEM may have a variable Vβ signature between individuals.

Taken into consideration overall, these data would appear to indicate that HLA class II polymorphisms cannot be the sole reason for differences observed in responses to SPEM between individuals, as individuals themselves appear to differ in their responses from day to day. This also suggests that from HLA types investigated there is no one HLA type which is either protective against SPEM, or which may lead to more severe disease such as ARF. This may indicate that other factors such as TcR Vβ polymorphisms could contribute to the effects seen. It should be considered that the general health of the donor may have day to day influences on Vβ responses to SPEM, and therefore severity of disease. Finally it cannot be ruled out that differences in experimental conditions from day to day contribute in part to the variation seen, although these experimental conditions are not likely to be completely responsible for the wide variation seen. Repetitions of the same donor on the same day (Figure 6.18) indicate that inter-experimental differences do not play a major role in variation observed. Further work along this line would need to be conducted to further clarify these results. This could include repeats of more donors on the same day or experiments conducted in a wider range of HLA typed donors. Multiplex PCR could be used to assess Vβ expansion in response to SPEM, possibly in addition to flow cytometric analysis to further characterise the Vβ signature of SPEM.

6.5 Conclusions

SPEC, L and M bind HLA class II in a zinc dependent manner, with SPEC and M demonstrating dosage dependent binding. SPEC and SPEM binding to HLA class II is blocked by TDR31.1, an anti-HLA class II β chain antibody, indicating high affinity β chain binding.

Possible α chain binding exists for all three toxins, reinforcing recent evidence of a low affinity binding 'interface' in SPEC [56].

SPEM causes widely varying Vβ responses between individuals of the same and different HLA types, and between individuals themselves. These Vβ responses are not necessarily restricted to the characterised Vβ signature of SPEM. HLA polymorphisms may play a role in these variations, but cannot form the entire basis for the observed differences.

7. Final Discussion and Future Work

The classical bacterial superantigens of *S. aureus* and *S. pyogenes* which have established roles in staphylococcal and streptococcal toxic shock syndrome and staphylococcal food poisoning, as well as hypothesised roles in a wide spectrum of disease including Kawasaki's Disease, acute rheumatic fever, atopic dermatitis, rheumatoid arthritis, psoriasis and autoimmunity [8, 9, 141, 168, 205]. Many superantigens are biologically active at very low concentrations, SMEZ1 for example induces half maximal proliferation of T cells at concentrations as low as 0.08pg/ml [178]. Additionally superantigens are serologically very diverse [8, 26]. These two issues make it difficult to study bacterial superantigens in the laboratory and in a clinical setting. Superantigens are usually detected in an indirect fashion, either by the detection of SAg antibodies in sera, or looking for Vβ specific changes in T cell repertoire [97, 161, 164, 178]. Few assays exist solely for the detection of the proteins themselves due to the limited commercial availability of SAg antibodies. Assays which do exist are usually directed at the detection of one particular superantigen, not a range. Changes in T cell repertoire are difficult to interpret with confidence as they vary with species [206, 207], between individuals of the same species [65-67]and the context in which the superantigen is acting in terms of factors such as APC density [208, 209], concentration of toxin [178] or HLA class II expression level [144]. Therefore a generalised capture molecule for the detection of superantigens would be a useful tool in both a laboratory and diagnostic setting. Such molecules could also hold potential for the development of novel therapeutic agents for treatment of SAg mediated disease. The HLA class II molecule is a logical starting point as this is the definitive receptor for bacterial superantigens [8, 210]. Therefore the primary aim of this work was to demonstrate binding of a range of superantigens using soluble HLA class II as a capture molecule. To undertake these experiments a panel of different HLA class II structures (whole molecules or conformationally intact partial-structures) and a range of superantigens exemplifying different modes of interaction with HLA class II are required.

A major challenge to this work has been the generation of sufficient quantities of different HLA class II types in solution to allow assays of binding to be performed. For the purposes of this research it was decided to attempt to purify HLA class II using affinity purification of HLA homozygous B cell membrane preparations. This method was chosen based on the wide range of HLA class II types which would be available [182], as well as the potential for purification of large quantities of HLA class II which would be required in the development of SAg capture assays. Relative ease of storage of HLA class II purified using this method was also considered an advantage. However attempts to purify HLA class II from B cell membranes were

ultimately unsuccessful despite several modifications to technique described in Chapter 4. Possible reasons for the failure of technique include degradation of monoclonal antibody used for affinity purification, or incomplete coupling of antibody to columns leading to elution of antibody. Alternatively HLA class II may not have been solubilised from B cell membrane preparations. Other techniques which could have been used for the generation of HLA class II structures were expression of recombinant complete or partial proteins in bacteria, yeast or insect cell lines. The relative merits and disadvantages of these techniques are outlined in Chapter 4 and Table 4.1.

A range of recombinant streptococcal superantigens were expressed (SPEA, C, L and M) and successfully conjugated (SPEC, L and M). The functional properties (as SAgs) of these conjugates were confirmed in Chapter 6. Unfortunately it was not possible to express the staphylococcal superantigen SEJ despite considerable modification to technique and troubleshooting. It is possible that the DNA amplified with primers designed based on the *sej* sequence was in fact non-coding DNA, however as all sequencing attempts failed this cannot be confirmed. Possible problems with the batch of TOP10 cells used giving rise to a large number of 'false' positive colonies also exist. This superantigen has not until very recently been successfully cloned and characterised [177], despite first being identified in 1998 [176]. Together with the difficulties expressing this toxin described in Chapter 3 this suggests that the gene may be particularly difficult to clone. The sequence may form secondary structures due to internal palindromes for example, or the plasmid may be deleted by intracellular mechanisms of *E. coli* [179].

Despite the difficulties encountered in purifying HLA class II, it was possible to undertake binding assays with HLA-DR15 stocks. These ELISA based assays showed detectable binding of a range of superantigens using HLA-DR15 as a capture molecule. These superantigens included SPEA, which reportedly preferentially binds HLA-DQ over HLA-DR [67] as well as SEA which utilises both the high affinity and low affinity binding sites of HLA-DR [64]. This showed that HLA-DR as an intact peptide loaded molecule can be used in a cell free system at a concentration of 3µg/ml and sufficiently broad specificity to bind a range of SAgs with differing binding modes. Zinc dependent binding of SPEC, SPEA, SPEM and to a lesser extent SEA was also demonstrated using these assays, reinforcing evidence that these superantigens bind HLA class II in a zinc dependent manner [24, 26, 32, 45, 58, 60, 61, 211]. Further, EDTA chelation reduced binding of all but SEA, suggesting that EDTA could be used as an elution agent in further assays such as affinity columns or beads.

The next phase for this work would most likely be to modify HLA class II structures to capture superantigens with broad specificity and high affinity. A structure based design approach could be used for this, identifying features of superantigen-HLA class II interfaces using software

such as GRASP (Dept of Biochemistry and Molecular Biophysics of Columbia University) [212] and QUANTA (Accelrys Cambridge UK). These softwares would help to identify important residues in the HLA class II α and β chains for superantigen binding, and thus identify targets for mutagenesis to increase affinity and broaden specificity for SAgs. A peptide repertoire based on the structure of HLA class II could then be designed with these sites in mind and surface plasmon resonance used to measure the affinities of these peptides for superantigens. The peptides could also be used in ELISA based assays as described in Chapter 5, or other cell free environments such as columns or magnetic beads.

As part of this project expression of SPEC and more notably the relatively under-explored toxins SPEL and SPEM has allowed the further analysis of these toxins in terms of Vβ specificity, outlined in Chapter 3. SPEC interestingly showed expansions in Vβ12 as expected, as well as Vβ8 which has not previously been documented to respond to SPEC [7]. TcR side chains are very important in binding of SPEC to Vβ regions, therefore it has been indicated previously that SPEC-Vβ interactions are particularly sensitive to mutations in the Vβ region [201]. This suggests there may be similarities between Vβ8 and Vβ12 at sites important for SPEC binding which are not apparent by phylogenetic analysis [79]. Until crystal data is available for SPEC complexed with TcR Vβ it is not possible to speculate any further on this. SPEL and SPEM are reported to expand the same Vβs - 1, 5.1 and 23 [58, 164]. Data presented in Chapter 3 indicates this is the case, with both SPEL and SPEM showing expansions in Vβs 1, 5.1 and 23 to differing extents. Interestingly the greatest proportions for all Vβs were seen at low concentrations of both toxins, suggesting a high level of toxicity at concentrations higher than 1μg/ml. SPEL seemed to preferentially expand Vβs 1 and 5.1, whilst SPEM preferentially expanded Vβs 1 and 23, suggesting that in *S. pyogenes* strains which carry both toxin genes, the two may work synergistically to expand large subsets of T cells. It has been suggested that HLA haplotype restrictions may have driven the evolution of *S. aureus* and *S. pyogenes* to carry multiple SAg genes [66], these data may reinforce this hypothesis. SPEM also showed a spreading TcR Vβ signature with increasing stimulus strength, consistent with the findings of Llewelyn *et al* [73].

Work described in Chapters 5 and 6 further characterised the binding mechanisms of SPEC, L and M. Chapter 5 described how addition of zinc increased binding of SPEC and SPEM to HLA class II in an ELISA based assay, reinforcing evidence that SPEC binds HLA class II zinc dependently with high affinity [24] and the hypothesis that SPEM utilises the β chain binding site in some way [58]. WT51 B cells were used in Chapter 6 to assess SAg-HLA binding. Again addition of zinc was required for detectable binding of SPEC, L and M to HLA class II. Competition assays between PE conjugated SAg and anti-class II antibodies indicated HLA class II β chain binding by SPEC, which is to be expected as SPEC contains two high affinity β chain binding sites [24, 32]. However L243 reduced binding of SPEC by 45.3%. This could

simply be attributed to stearic hindrance due to the size of immunoglobulin and interference with the superantigen binding site on the HLA class II β chain, but is in keeping with the recent observation of SPEC binding to the HLA class II α chain [56]. Binding assays in Chapter 5 (Figures 5.3b and 5.4b) showed that even in the absence of zinc, and chelation of zinc by EDTA, some binding of SPEC by HLA class II was detected. This residual binding may be attributed to α chain involvement in SPEC binding as reinforced by data in Chapter 6.

Binding of HLA class II to SPEM was markedly reduced by both TDR31.1 and L243 suggesting β chain binding by this toxin, as well as possible α chain involvement. This is consistent with evidence that SPEM belongs to the same group of SAgs as SPEC (Group IV, Figure 1.3 [26], as well as evidence that SPEM bind HLA class II in a high affinity zinc dependent manner [58]. This is also consistent with earlier findings in this research that SPEM requires zinc for binding to HLA class II. Inhibition of binding of SPEM by L243 may indicate the presence of a low affinity 'interface' in these superantigens similar to that recently identified in SPEC [56]. SPEL binding was reduced by TDR31.1 to a smaller extent. L243 did not inhibit binding of SPEL. This may indicate binding to the HLA-DQ α chain or that SPEL binding to the class II β chain adopts a conformation not antagonised by TDR31.1. Further study of these differences in SPEL and M interaction with HLA class II could be undertaken using site-directed mutagenesis, measurements of affinity by surface plasmon resonance and crystallography studies.

As anti HLA class II antibodies did not completely inhibit binding of any SAg some binding may be non-functional. This possibility could be explored by testing the ability of SAgs bound to APCs in the presence of saturating concentrations of class II antibody to activate purified T cells or T cell clones.

A final intriguing observation which has arisen from this project has been the observation of variation in superantigen responses both between individuals and between days in the same donor. This was particularly pronounced in response to SPEM (Chapters 3 and 6) but was also noted with SPEL in Chapter 3. Previously it has been shown that polymorphisms in HLA-DQ and HLA-DR can influence the presentation of superantigens to the TcR [65-67]. A similar effect could explain variation in responses to SPEM. The responses to SPEM in 5 healthy donors of known HLA type were investigated in triplicate in Chapter 6. These donors were grouped based on criteria described in Chapter 6 methods. Interestingly, although overall trends between the groups were similar (Figure 6.13) showing dosage dependent expansions of Vβs 5.1 and 23, and to a lesser extent Vβ1, these trends not only differed from results observed in Chapter 3 (Figures 3.8-10), where Vβ1 was the primary Vβ targeted by SPEM, but also marked variation between individual donors within groups were also apparent, which was consistent with findings in Chapter 3. No clear correlation was apparent between members of the same

groups. Therefore it could be the case that HLA polymorphisms appear to have no discernable effect on the Vβ responses to SPEM. However it should also be noted that grouping of donors was conducted based on very limited sites within the HLA-DR β chain as grouping on a more complicated scale was not possible. This means differences in HLA-DR β chain may be more pronounced between HLA types studied than is apparent from these sites, which may impact upon superantigen binding, therefore no firm conclusions on the influence of HLA type on SPEM responses can be drawn. Of particular note is the variation in responses in the same donor at different times. An additional repeat of one donor on the same day (Figure 6.18) indicated that experimental differences were not causing this variation. The variation was noticed to different extents in all donors, some more marked than others (Donor 3 for example) and would appear to suggest that time point plays as critical a role in the host response to SPEM as HLA type. This may indicate that other factors are involved in the host response, such as the hosts' general state of health, or environmental factors. Other genetic factors may also play a part, for example the TcR Vβ genes, or other co-stimulatory immune molecules although these should only account for the variation between individuals, not days.

Further research could be conducted into SPEM responses in more HLA typed donors, to allow grouping based on DQ α and β chains in addition to DR β chains to further investigate the mechanism of SPEM binding and the influence HLA polymorphisms have on the Vβ response to this toxin. Investigations could also be carried out as to the effect time or day has on the responses to SPEM. Vβ specific proliferation could be measured by both multiplex PCR and flow cytometry to obtain a clearer overall picture of Vβ signatures. Additionally responses to SPEM should be investigated with if possible the full range of available Vβ antibodies to determine if expansions are seen elsewhere in the Vβ repertoire. A final possibility would be to study these responses with a wider range of novel superantigens, as studies of this nature are usually conducted with the more classical and well characterised superantigens [65-67].

This research has provided some interesting conclusions. SPEA primarily targets Vβ14, whilst the Vβ repertoire of SPEC seems to be conflicting and inconclusive. SPEL and SPEM appear to expand Vβs 1, 5.1 and 23 with differing hierarchies, suggesting possible synergy between the two toxins. Soluble HLA class II can be used to detect a range of superantigens bearing different binding modes in solution. This binding is zinc dependent and reduced by EDTA. HLA-DR has a sufficiently broad specificity and affinity to detect all these superantigens. SPEC, SPEL and SPEM bind HLA class II on the surface of B cells with zinc dependence and high affinity, there is also a possibility of low affinity binding as well. Finally, TcR Vβ responses to SPEM differ considerably between donors, and between the same individual at different times. This variation cannot be entirely attributed to HLA differences.

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Appendices

Appendix 1 *DRB1* protein sequences

(showing only positions 1-100) *DRB1* alleles used in this study are highlighted in grey. Sequences downloaded from IMGT/HLA database [39]

```
Prot. Pos.                10         20         30         40         50         60         70         80         90        100
                           |          |          |          |          |          |          |          |          |          |
DRB1*010101       GDTRPRFLWQ LKFECHFFNG TERVRLLERC IYNQEESVRF DSDVGEYRAV TELGRPDAEY WNSQKDLLEQ RRAAVDTYCR HNYGVGESFT VQRRVEPKVT
DRB1*010102       *****----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----******
DRB1*010103       *****----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----******
DRB1*010201       ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----AV---- ----------
DRB1*010202       *****----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----AV**** **********
DRB1*010203       *****----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----AV---- ----******
DRB1*010204       *****----- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----AV---- ----******
DRB1*0103         ---------- ---------- ---------- ---------- ---------- ---------- ------I--D E--------- ---------- ----------
DRB1*0104         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ------N--- -----V---- ----------
DRB1*0105         *****----- ---------- ---------- ---------- ----R----- ---------- ---------- ---------- ---------- ----******
DRB1*0106         *****----- ---------- ---------- ---------- ---------- ---------- ---------- A--------- -----V---- ----******
DRB1*0107         *****----E ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----******
DRB1*0108         *****----- ---------- ---------- ------Y--- ---------- ---------- ---------- ---------- ---------- ----******
DRB1*0109         *****----- ---------- ---------- ---------- ---------- ---------- ---------- A--------- ---------- ----******
DRB1*0110         *****----- ---------- ---------- ---------- ---------- ---------- ---------- K--------- ---------- ----******
DRB1*0111         *****----- ---------- ---------- ---------- ---------- ---------- ---------- ------N--- ---------- ----******
DRB1*0112         *****----- ---------- ---------- ---------- ---------- ---------- ---------- -L-------- ---------- ----******
DRB1*0113         ---------- ---------- ----QF---L F--------- ---------- ---------- ---------- ---------- ---------- ----------
DRB1*0114         *****----- ---------- ---------- ---------- ---------- ------A--H ---------- ---------- ---------- ----******
DRB1*0115         *****----- ---------- ---------- ---------- ---------- ---------- ------I--- A--------- ---------- ----******
DRB1*0116         *****----- ---------- ---------- ---------- ---------- ---------- ---------- K-GR--N--- ---------- ----******
DRB1*0117         *****----- ---------- ---------- ---------- ---------- ---------- ---------- ---E------ ---------- ----******
DRB1*0118         *****----- ---------- ---------- ---------- ------F--- ---------- ---------- ---------- ---------- ----******
DRB1*0119         *****----- ---------- ---------- ---------- ---------- ---------- ---------- -----N---- ---------- ----******
DRB1*030101       --------EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- -----H----
DRB1*030102       --------EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- -----H----
DRB1*030103       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*030104       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*030105       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*030106       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*030201       --------EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- K-GR--N--- ---------- -----H----
DRB1*030202       --------EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- K-GR--N--- ---------- -----H----
DRB1*0303         *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- K-GR--N--- -----V---- ----******
```

```
Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*0304       *****---EY STS------- -----Y-D-Y FH-------- ------F--- ---------- ---------- K-GR--N--- -----V---- -----H----
DRB1*030501     *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- ---------- ----******
DRB1*030502     *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- ---------- ----******
DRB1*0306       --------EY STS------- -----Y-D-Y FH----N--- ---------- ---------- ---------- K-GR--N--- -----V---- -----H----
DRB1*0307       --------EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- -----H----
DRB1*0308       --------EY STS------- -----Y-D-Y FH----N--- ------F--- -------E-- ---------- K-GR--N--- -----V---- -----H----
DRB1*0309       *******-EY STS------- -----Y-D-Y FH-R--N--- ------F--- ---------- ---------- K-GR--N--- ---------- ---*******
DRB1*0310       --------EY STS------- -----Y-D-Y FH----N--- ------F--- ------A--H ---------- K-GR--N--- -----V---- -----H----
DRB1*0311       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GQ--N--- -----V---- ----******
DRB1*0312       *******-EY STS------- -----Y-D-Y FH----N--- ------F--- ------S--- ---------- K-GR--N--- -----V-*** **********
DRB1*0313       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------S ---------- K-GR--N--- -----V---- ----******
DRB1*0314       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR------ ---------- ----******
DRB1*0315       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR------ -----V---- -----H----
DRB1*0316       *****---EY STS------- -----Y-D-Y FH----N--- ------FW-- ---------- ---------- K-GR--N--- -----V**** **********
DRB1*0317       *****---EY STS------- -----F-D-Y F-----Y--- ---------- R--------- ---------- K-GQ--N--- ---------- ----******
DRB1*0318       *****---EY STS------- -----Y-D-Y FH----N--- ----R-F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0319       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ------I--- K-GR--N--- -----V---- ----******
DRB1*0320       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- ----AV---- ---*******
DRB1*0321       *****---EY STS------- -----F-D-Y FH----F--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0322       *****---EY STS------- -----Y-D-Y FD----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0323       *****---EY STS------- -----Y-D-Y FH-R--N--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0324       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ---------- K-GQ--N--- -----V---- ----******
DRB1*0325       *****---EY STS------- -----Y-D-Y FH----Y--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0326       *****---EY STS------- -----Y-D-Y FH----NA-- ---------- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0327       *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- K-GQ--N--- ---------- ----******
DRB1*0328       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- -K-------- ---------- K-GR--N--- -----V---- ----******
DRB1*0329       *****---EY STS------- -----F---Y FH----N--- ------F--- ---------- ---------- K-GR--N--- ---------- ----******
DRB1*0330       *****---EY STS------- -----Y-D-Y FH----F--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0331       *****---EY STS------- -----Y-D-Y FH----Y--- ---------- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0332       *****---EY STS------- -----YVD-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0333       *****---EY STS------- -----Y-D-Y FH----NA-- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0334       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------- K-GR-NN--- -----V---- ----******
DRB1*0335       *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ---------- K-GQ--N--- ---------- ----******
DRB1*0336       *****---EY STS------- -----Y-D-Y FHK---N--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*0337       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- R--------- ---------- K-GR--N--- -----V---- ----******
DRB1*0338       *****---EY STS------- -----F---Y FH----N--- ---------- ------V--S ---------- K-GR--N--- ---------- ----******
DRB1*0339       *****---EY STS------- -----YRD-Y FH----N--- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*040101     --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- K--------- ---------- -----Y-E--
DRB1*040102     *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- K--------- ---------- ----******
DRB1*040103     *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- K--------- ---------- ----******
DRB1*0402       --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ------I--D E--------- -----V---- -----Y-E--
DRB1*040301     --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ -----V---- -----Y-E--
DRB1*040302     *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ -----V---- -----Y-E--
DRB1*040303     *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ -----V---- ----******
DRB1*0404       --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---------- -----V---- -----Y-E--
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*040501       --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- ---------- ---------- -----Y-E--
DRB1*040502       ******--E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- ---------- ---------- ----******
DRB1*040503       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- ---------- ---------- ----******
DRB1*040504       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- ---------- ---------- ----******
DRB1*040505       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- ---------- ---------- ----******
DRB1*040601       --------E- V-H------- -----F-D-Y F-H------- ---------- ---------- ---------- ---E------ -----V---- -----Y-E--
DRB1*040602       --------E- V-H------- -----F-D-Y F-H------- ---------- ---------- ---------- ---E------ -----V---- -----Y-E--
DRB1*040701       --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ ---------- -----Y-E--
DRB1*040702       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ ---------- ---*******
DRB1*040703       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ ---------- ----******
DRB1*0408         --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---------- ---------- -----Y-E--
DRB1*0409         --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- K--------- ---------- -----Y-E--
DRB1*0410         --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- ---------- -----V---- -----Y-E--
DRB1*0411         --------E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- ---E------ -----V---- -----Y-E--
DRB1*0412         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ------I--D ---L------ -----V---- ----******
DRB1*0413         ********** **H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- K--------- -----V---- **********
DRB1*0414         ********** ***------- -----F-D-Y F-H---Y--- ---------- ---------- ------I--D E--------- -------*** **********
DRB1*0415         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- -------E-- ------F--D ---------- -----V---- -----Y-E--
DRB1*0416         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- --------Q- ---------- K--------- ---------- -----Y-E--
DRB1*0417         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- ---E------ ---------- ----******
DRB1*0418         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ------I--D ---L------ -----V---- ----******
DRB1*0419         ******--E- V-H------- -----F-D-Y F-H------- ---------- ---------- ---------- ---------- ---------- ----******
DRB1*0420         ********** ***------- -----F-D-Y F-H------- ---------- ---------- ---------- ---E------ ------**** **********
DRB1*0421         ********E- V-H------- -----F-D-Y F-H------- ---------- ---------- ---------- K--------- ---------- -*********
DRB1*0422         ********E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- K-GR--N--- -----V---- **********
DRB1*0423         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---------- -----V-R-- ----******
DRB1*0424         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------R ---------- ---------- ----******
DRB1*0425         *******-E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ------F--D ---L------ -----V-*** **********
DRB1*0426         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- -------T-- ---------- K--------- ------**** **********
DRB1*0427         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ ----AV---- -*********
DRB1*0428         *****---E- V-H------- -----F-D-Y F-H---Y--- ------F--- ------S--- ---------- ---------- ---------- ----******
DRB1*0429         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- M-----S--- ---------- ---------- ---------- ----******
DRB1*0430         *****---E- V-H------- -----F-D-Y F-H---Y--- --------V- ------S--- ---------- ---------- ---------- ----******
DRB1*0431         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---L------ ---------- -----Y-E--
DRB1*0432         *******-E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- -Q--------- -----V-*** **********
DRB1*0433         *****---E- V-H------- -----F-D-Y F-H---Y-H- ---------- ---------- ---------- K--------- ------**** **********
DRB1*0434         *****---E- V-P------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- K--------- ---------- -----Y-E--
DRB1*0435         *****---E- V-H------- -----F-D-Y F-H---Y--- ------F--- ---------- ---------- K--------- ---------- -*********
DRB1*0436         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ------F--D ---------- -----V---- ----******
DRB1*0437         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------D E--------- -----V---- ----******
DRB1*0438         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ------I--- K--------- ---------- ----******
DRB1*0439         *****---E- V-H------- -----F-D-Y F-H---Y--- -----D---- ---------- ---------- ---E------ -----V---- ----******
DRB1*0440         *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- -------G-- ---------- ---------- -----V---- ----******
DRB1*0441         *****---E- V-H------- -----F-D-Y F-H---N--- ---------- ---------- ---------- ---E------ -----V---- ---*******
DRB1*0442         *****---E- V-H------- -----F-D-Y F-H---Y--- ------F--- ---------- ---------- ---------- -----V---- ----******
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*0443       *****---E- V-H------- -----F-D-Y F-H---Y--- ------F--- ---------- ---------- ---------- ---------- ---*******
DRB1*0444       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ------N--- -----V---- --********
DRB1*0445       *****---E- V-H------- --P--F-D-Y F-H---Y--- ---------- ------S--- ---------- ---------- ---------- ----******
DRB1*0446       *****---E- V-H------- -----F-D-Y F-H------- ---------- -----A---- ---------- ---E------ -----V---- ----******
DRB1*0447       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ------F--D ---------- ---------- ----******
DRB1*0448       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- --S------- ---------- ----******
DRB1*0449       *****---E- I-H------- -----F-D-Y F-H------- ---------- ---------- ---------- ---E------ -----V---- ----******
DRB1*0450       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E---H-- -----V---- ----******
DRB1*0451       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ Y----V---- ----******
DRB1*0452       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ -----V-R-- ----******
DRB1*0453       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ------I--D ---------- -----V---- ----******
DRB1*0454       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ------F--D ---L------ ---------- ----******
DRB1*0455       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---L------ -----V---- ----******
DRB1*0456       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ------I--- ---------- -----V---- ----******
DRB1*0457       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--N ---------- ---------- ---------- ----******
DRB1*0458       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------D ---L------ -----V---- ----******
DRB1*0459       *****---E- V-H------- -----F-D-Y F-H---Y--- ------F--- ---------- ---------- ---E------ -----V---- ----******
DRB1*0460       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- --N------- ---E------ -----V---- ----******
DRB1*0461       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- -------E-- ---------- ---------- ---------- ----******
DRB1*0462       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------A--H ---------- K--------- ---------- ----******
DRB1*0463       *****---E- V-H------- -----F-D-Y F-----Y--- ---------- ---------- ---------- K--------- ---------- ----******
DRB1*0464       *****---E- V-Y------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- K--------- ---------- ----******
DRB1*0465       *****---E- V-H------- -----F-D-Y F-H------- ---------- ---------- ---------- W--E------ -----V---- ----******
DRB1*0466       *****---E- A-S------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- K--------- ---------- ----******
DRB1*0467       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------S--- ---------- K--------- -----V---- ----******
DRB1*0468       *****---E- V-H------- -----F-D-Y F-H------- ---------- ---------- ---------- ---------- -----V---- ----******
DRB1*0469       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ------A--H ---------R ---E------ ---------- ----******
DRB1*0470       *****---E- V-H------- -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---------- Y----V---- ----******
DRB1*0471       *****---E- V-H------R -----F-D-Y F-H---Y--- ---------- ---------- ---------- ---E------ -----V---- ----******
DRB1*070101     ---Q------ G-YK------ ----QF---L F-----F--- ---------- ------V--S ------I--D --GQ---V-- ---------- -----H-E--
DRB1*070102     *****----- G-YK------ ----QF---L F-----F--- ---------- ------V--S ------I--D --GQ---V-- ------**** **********
DRB1*0703       *****----- G-YK------ ----QF--SL F-----F--- ---------- ------V--S ------I--D --GQ---V-- ------**** **********
DRB1*0704       *****----- G-YK------ ----QF---L F-----F--- ---------- ------V--S ------I--D --GQ--N--- ---------- -----H-E--
DRB1*0705       *****----- G-YK------ ----QF---L F-----F--- ---------- ------V--S ---R--I--D --GQ---V-- ---------- **********
DRB1*0706       *****----- G-YK------ ----QF---L F-----F--- ---------- ------A--- ------I--D --GQ---V-- ---------- ----******
DRB1*0707       *****----- G-YK------ ----QF---L F-----F--- ---------- ----W-V--S ------I--D --GQ---V-- ---------- -*********
DRB1*0708       *****----- G-YK------ ----QF---L F-----V--- ---------- ------V--S ------I--D --GQ---V-- ---------- ----******
DRB1*0709       ---Q------ G-YK------ ----QF---F F-----F--- ---------- ------V--S ------I--D --GQ---V-- ---------- -----H-E--
DRB1*0710N      *****----- G-YK------ ----QF---L LX
DRB1*0711       *****----- G-YK------ ----QF---L F-----F--- ---------- ------V--S ------I--D --GQ------ ---------- ----******
DRB1*0712       *****----- G-YK------ ----QF---L F-----F--- ---------- ------I--S ------I--D --GQ---V-- ---------- ----******
DRB1*0713       *****----- G-YK------ ----QF---L F-----F--- ---------- ----L-V--S ------I--D --GQ---V-- ---------- ----******
DRB1*0714       *****----- G-YK------ ----QF---L F---G-F--- ---------- ------V--S ------I--D --GQ---V-- ---------- ----******
DRB1*0715       *****----- G-YK------ K---QF---L F-----F--- ---------- ------V--S ------I--D --GQ---V-- ---------- ----******
DRB1*080101     --------EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------F--D ---L------ ---------- -----H----
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*080102      *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------F--D ---L------ ---------- ----******
DRB1*080103      --------EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------F--D ---L------ ---------- -----H----
DRB1*080201      --------EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---L------ ---------- -----H----
DRB1*080202      *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---L------ ---------- -----H----
DRB1*080203      *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---L------ ---------- -*********
DRB1*080302      --------EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D ---L------ ---------- -----H----
DRB1*080401      --------EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---L------ -----V---- -----H----
DRB1*080402      ********** *******--- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---L------ -----V---- ---*******
DRB1*080403      *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---L------ -----V---- ----******
DRB1*080404      *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---L------ -----V---- ----******
DRB1*0805        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------F--D ---------- ------**** **********
DRB1*0806        --------EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------F--D ---L------ -----V---- -----H----
DRB1*0807        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------V--- ------F--D ---L------ ---------- -----H----
DRB1*0808        *******-EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------A--H ------F--D ---L------ -------*** **********
DRB1*0809        *****---EY STG--Y---- -----F-D-Y FH----F--- ---------- ---------- ------F--D ---L------ ---------- ----******
DRB1*0810        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D ---L------ -----V---- ----******
DRB1*0811        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------A--- ------F--D ---L------ ---------- -----H----
DRB1*0812        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D ---L------ ----AV---- ----******
DRB1*0813        --------EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------- ---------D ---L------ ---------- -----H----
DRB1*0814        *****---EY SRG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D ---L------ ---------- -----H----
DRB1*0815        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------H ------I--D ---L------ ---------- ----******
DRB1*0816        *****---EY STG--Y---- -----F-D-Y F-----D--- ---------- ------S--- ------F--D ---L------ ---------- ----******
DRB1*0817        *****---EY STG--Y---- -----F-D-Y F-----Y--- ------F--- ------S--- ------F--D ---L------ ------**** **********
DRB1*0818        --------EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D ---------- ---------- -----H----
DRB1*0819        ******--EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------I--- ------I--D ---L------ ---------- -*********
DRB1*0820        *****---EY STS------- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---L------ -----V---- ----******
DRB1*0821        *****---EY SMG--Y---- -----F-D-Y FH----F--- ---------- ---------- ------F--D ---L------ ---------- ----******
DRB1*0822        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------F--D ---L------ ----AV---- ----******
DRB1*0823        *****---EY STG--Y---- -----F-D-Y F-----Y--- ----R----- ------S--- ------I--D ---L------ ---------- ----******
DRB1*0824        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---------- ---------- ---*******
DRB1*0825        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------V--S ------I--D ---------- ---------- ----******
DRB1*0826        *****---EY STG--Y---- ----LF-D-Y F-----Y--- ---------- ------S--- ------F--D ---L------ ---------- ----******
DRB1*0827        *****---EY STG--Y---- -----F-D-Y F--R--Y--- ---------- ------S--- ------I--D ---L------ ---------- ----******
DRB1*0828        *****---EY STG--Y---- -----F-D-Y F-----Y--- ------F--- ---------- ------F--D ---L------ -----V---- ----******
DRB1*0829        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D ---E------ ---------- ----******
DRB1*0830        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ---------- ------I--D ---L------ ---------- ----******
DRB1*0831        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- -------E-- ------F--D ---------- -----V---- ----******
DRB1*0832        *****---EY STG--Y---- ---------H FH----LL-- ---------- ------S--- ------I--D ---L------ ---------- ----******
DRB1*0833        *****---EY STG--Y---- -----F-D-Y F-----Y--- -------W-- ------S--- ------I--D ---L------ ---------- ----******
DRB1*0834        *****---EY STG--Y---- -----F-D-Y F-----Y--- ---------- ------V--S ------I--D ---L------ ---------- ----******
DRB1*090102      ---Q----K- D--------- -----Y-H-G ------N--- ---------- ------V--S ------F--R ---E---V-- ---------- -----H-E--
DRB1*090103      *****---K- D--------- -----Y-H-G ------N--- ---------- ------V--S ------F--R ---E---V-- ---------- ----******
DRB1*0902        *****---K- D--------- -----Y-H-G ------N--- ---------- ---------- ------F--R ---E---V-- ---------- ----******
DRB1*0903        *****---K- D--------- -----Y-H-G ------N--- ---------- ------V--S ------F--D ---E---V-- ---------- ----******
DRB1*0904        *****---K- D--------- -----Y-H-G ------N--- ---------- ------V--S ---------R ---E---V-- ---------- ----******
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*0905       *****---K- D--------- -----Y-H-G ------N--- ---------- ------V--S ------F--R ---E------ ---------- ----******
DRB1*0906       *****---K- D--------- -----Y-H-G ------N--- ---------- ------V--S ------F--R ---E---V-- -----V---- ----******
DRB1*100101     --------EE V--------- ---------R VH----YA-Y ---------- ---------- ---------R ---------- ---------- -----Q----
DRB1*100102     *****---EE V--------- ---------R VH----YA-Y ---------- ---------- ---------R ---------- ---------- ----******
DRB1*1002       *****---EE V--------- ---------R VH----YA-Y ---------- ---------- ---------R ---------- -----V---- ----******
DRB1*110101     --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ---------- -----H----
DRB1*110102     --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ---------- -----H----
DRB1*110103     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*110104     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*110105     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*110106     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*110107     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*110201     --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------I--D E--------- -----V---- -----H----
DRB1*110202     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------I--D E--------- -----V---- ----******
DRB1*1103       --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D E--------- -----V---- -----H----
DRB1*110401     --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- -----V---- -----H----
DRB1*110402     --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- -----V---- -----H----
DRB1*110403     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- -----V---- ----******
DRB1*110404     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- -----V---- ----******
DRB1*1105       *****---EY STG------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ---------- -----H----
DRB1*110601     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ----AV---- ----******
DRB1*110602     ******--EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ----AV---- ----******
DRB1*1107       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ---------- K-GR--N--- -----V---- ----******
DRB1*110801     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ---------D ---------- ---------- -----H----
DRB1*110802     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ---------D ---------- ---------- -----H----
DRB1*1109       *****---EY STS------- -----F-D-Y FH----N--- ------F--- -------E-- ------F--D ---------- ---------- -----H----
DRB1*1110       *****---EY STS------- -----F-D-Y FH----F--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*111101     --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D E--------- ---------- ----******
DRB1*111102     --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D E--------- ---------- -----H----
DRB1*111201     *****---EY STS------- -----F-D-Y F-----F--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*111202     *****---EY STS------- -----F-D-Y F-----F--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*1113       --------EY STS------- -----F-D-Y FH----F--- ------F--- -------E-- ---------R ---------- -----V---- -----H----
DRB1*111401     --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------I--D E--------- ---------- -----H----
DRB1*111402     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------I--D E--------- ---------- ----******
DRB1*1115       --------EY STS------- -----F-D-Y F-----DL-- ------F--- -------E-- ------F--D ---------- ---------- -----H----
DRB1*1116       *****---EY STS------- -----F-D-Y FH----N--- ------F--- -------E-- ------I--D E--------- -----V---- ----******
DRB1*1117       --------EY STS------- -----F-D-Y FH----F--- ---------- -------E-- ---------R ---E------ -----V---- -----H----
DRB1*1118       ******--EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------I--D ---------- -----V---- ----******
DRB1*111901     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------I--D ---------- ---------- -----H----
DRB1*111902     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------I--D ---------- ---------- ----******
DRB1*1120       *******-EY STS------- -----F-D-Y FH----N--- ------F--- -------E-- ------I--D E--------- ---------- --********
DRB1*1121       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------I--D E--------- ----AV---- -----H----
DRB1*1122       *****---E- V-H------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- -------*** **********
DRB1*1123       ****----EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---L------ -------**** **********
DRB1*1124       ******--EY STS------- -----F-D-Y F-----D--- ------F--- -------E-- ------F--D ---------- ---------* **********
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*1125       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---L------ -----V---- ----******
DRB1*1126       *******-EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ---------- ---------- -------*** **********
DRB1*112701     ******--EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ------N--- -------*** **********
DRB1*112702     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ------N--- ---------- ----******
DRB1*1128       *****---EY STS------- -----F-D-Y F-----N--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*1129       *****---EY STS------- -----F-D-Y F--------- ------F--- -------E-- ------F--D ---------- ---------- -----H----
DRB1*1130       *****---EL --S------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*1131       --------EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-H ------I--D ---------- ---------- -----H----
DRB1*1132       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---V------ ---------- ----******
DRB1*1133       *******-EY STS------- -----F-D-Y F-----Y--- ------F--- -------ED- ------F--D ---------- ---------- ---*******
DRB1*1134       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ---------- ---------- -----V---- ----******
DRB1*1135       *******-EY STS------- -----F-D-Y F-----Y--- ------F--- -------ED- ------F--D ---------- -----V---- ---*******
DRB1*1136       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ---------D E--------- -----V---- ----******
DRB1*1137       *****---EY STS------- -----F-D-Y F-----Y--- ---------- -------E-- ------F--D ---------- ---------- ----******
DRB1*1138       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------EG- ------F--D ---------- -----V---- ----******
DRB1*1139       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- R------E-- ------F--D ---------- ---------- ----******
DRB1*1140       *****---EY STS------- -----F-D-Y FH----N--- ------F--- -------E-- ------F--D E--------- -----V**** **********
DRB1*1141       ******--EY STS------- -----F-D-Y F-----Y--- ---------- -------E-- ------F--D E--------- -----V---- ----******
DRB1*1142       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ---------D ---------- -----V---- ----******
DRB1*1143       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- R------E-- ------F--D ---------- -----V---- ----******
DRB1*1144       *****---EY STS------- -----F-D-Y F-----YA-- ------F--- -------E-- ------F--D ---------- -----V---- ----******
DRB1*1145       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------I--D ---L------ ---------- ----******
DRB1*1146       *****---EY STS------- -----F-D-Y FH----Y--- ------F--- -------E-- ------F--D ---------- -----V---- ----******
DRB1*1147       *****---EY STS------- -----F-D-Y F--------- ------F--- -------E-- ------F--D ---------- ----AV---- ----******
DRB1*1148       *****---EY STS------- -----F-D-Y F-----Y--- ---------- -------E-- ------I--D E--------- -----V---- ----******
DRB1*1149       *****---EY STS------- -----F-D-Y F-----YA-- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*1150       *****---EY STS------- -----F-D-Y F-----YA-- ---------- R------E-- ------F--D ---------- -----V---- ----******
DRB1*1151       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D -Q-------- ---------- ----******
DRB1*1152       *****---EY STS------- -----F-D-Y FH----F--- ---------- -------E-H ---------R ---E------ -----V---- ----******
DRB1*1153       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--- K-GQ------ ---------- ----******
DRB1*115401     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---E------ -----V---- ----******
DRB1*115402     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D ---E------ -----V---- ----******
DRB1*1155       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ------F--D E--L------ -----V---- ----******
DRB1*1156       *****---EY STS------- -----F-D-Y F-----Y--- ---------- -------E-- ------F--D ---------- -----V---- ----******
DRB1*1157       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ---------D ---------- ----AV---- ----******
DRB1*1158       *****---EY STS------- -----F-D-Y F-----N--- ------F--- -------E-- ------F--D ---------- -----V---- ----******
DRB1*1159       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- -------E-- ------F--D E--------- -----V---- ----******
DRB1*1160       *****---EY STS------- -----F-D-Y F-----Y--- ------F-E- -------E-- ------F--D ---------- -----V---- ----******
DRB1*1161       *****---EY STS------- -----F-D-Y F-----Y--- N-----F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*1162       *****---EY STS------- -----F-D-Y FH----Y--- ------F--- -------E-- ------F--D ---------- ---------- ----******
DRB1*1163       *****---EY STS------- -----F-D-Y F--------- ------F--- -------E-- ------F--D E--------- -----V---- ----******
DRB1*1164       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------E-- ---------D ---L------ ---------- ----******
DRB1*116501     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------ED- ------I--D E--------- -----V---- ----******
DRB1*116502     *****---EY STS------- -----F-D-Y F-----Y--- ------F--- -------ED- ------I--D E--------- -----V---- ----******
DRB1*1166       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- M------E-- ------F--D ---------- ---------- ----******
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*120101       --------EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ------I--D ---------- ----AV---- -----H----
DRB1*120102       --------EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ------I--D ---------- ----AV---- -----H----
DRB1*120201       *****---EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ------F--D ---------- ----AV---- ----******
DRB1*120202       ******--EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ------F--D ---------- ----AV-*** **********
DRB1*120302       *****---EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ------I--D ---------- -----V---- ---*******
DRB1*1204         *****---EY STG--Y---- ---------H FH----LL-- ------F--- -------E-- ------I--D ---------- ----AV---- ----******
DRB1*1205         *****---EY STG--Y---- ---------H FH----FL-- ------F--- ------V--S ------I--D ---------- ----AV---- ----******
DRB1*1206         --------EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ------I--D ---------- ----AV---- -----H----
DRB1*1207         *****---EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ------I-GD ---------- ----AV---- -----H----
DRB1*1208         *****---EY STG--Y---- -----F---H FH----LL-- ------F--- ------V--S ------I--D ---------- ----AV---- ----******
DRB1*1209         *****---EY STG--Y---- ---------H FH----LL-- ------F--- ---------- ------I--D ---------- ----AV---- ----******
DRB1*1210         --------EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ------I--D ---------- ----AV---- -----H----
DRB1*1211         *****---EY STG--Y---- ---------H FH----LL-- ------L--- ------V--S ------I--D ---------- ----AV---- ----******
DRB1*1212         *****---EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ---------D ---------- ----AV---- ----******
DRB1*1213         *****---EY STG--Y---- ----L----H FH----LL-- ------F--- ------V--S ------F--D ---------- ----AV---- ----******
DRB1*1214         *****---EY STG--Y---- ---------H FH----F--- ------F--- ------V--S ------I--D ---------- ----AV---- ----******
DRB1*1215         *****---EY STG--Y---- ---------H FH----FL-- ------F--- ------V--S ------F--D ---------- ----AV---- ----******
DRB1*1216         *****---EY STG--Y---- ---------H FH----LL-- ------F--- ------V--S ------F--D ---------- ---------- ----******
DRB1*130101       --------EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E--------- -----V---- -----H----
DRB1*130102       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E--------- -----V---- ----******
DRB1*130103       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E--------- -----V---- ----******
DRB1*130201       --------EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E--------- ---------- -----H----
DRB1*130202       --------EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E--------- ---------- ---*******
DRB1*130203       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E--------- ---------- ----******
DRB1*130301       --------EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D K--------- ---------- -----H----
DRB1*130302       *******-EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D K--------- ---------- ----******
DRB1*1304         --------EY STS------- -----F-D-Y F-----Y--- ------F--- ------S--- ------I--D E--------- -----V---- -----H----
DRB1*130501       --------EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------F--D ---------- ---------- -----H----
DRB1*130502       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------F--D ---------- ---------- ----******
DRB1*1306         *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D ---------- -----V---- -----H----
DRB1*130701       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---------- ---------- ----******
DRB1*130702       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---------- ---------- ----******
DRB1*1308         *****---EY STS------- -----F-D-Y FH----F--- ---------- ---------- ------I--D E--------- -----V---- -----H----
DRB1*1309         ******--EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--- A--------- -----V---- -*********
DRB1*1310         *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D K--------- -----V---- -----H----
DRB1*1311         *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ---------- ------F--D ---------- -----V---- -----H----
DRB1*1312         *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D ---------- ---------- ----******
DRB1*1313         *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D ---L------ ---------- -*********
DRB1*131401       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ---------- ------F--D ---------- ---------- ----******
DRB1*131402       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ---------- ------F--D ---------- ---------- -----H----
DRB1*1315         ******--EY STS------- -----F---Y FH----N--- ------F--- ---------- ------I--D E--------- -----V---- ----******
DRB1*1316         *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E--------- -----D---- ----******
DRB1*1317         --------EY STG--Y---- -----F-D-Y F-----Y--- ------F--- ---------- ------I--D E--------- -----V---- -----H----
DRB1*1318         *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------F--D ---L------ -----V---- ----******
DRB1*1319         --------EY STS------- -----F---Y FH----F--- ---------- ---------- ------I--D E--------- -----V---- -----H----
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*1320       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ---------D E--------- -----V---- -----H----
DRB1*1321       --------EY STS------- -----F-D-Y F-----Y--- ------F--- ------S--- ------F--D ---------- ---------- -----H----
DRB1*1322       ****----EY STS------- -----F-D-Y F-----Y--- ------F--- ---------- ------I--D E--------- -----V---- ----******
DRB1*1323       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ---------- ------I--D E--------- ---------- ---*******
DRB1*1324       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ---------- ------F--D E--------- -----V---- ---*******
DRB1*1325       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ---------- ---------D ---------- -------*** **********
DRB1*1326       *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ------F--D ---------- ---------- ----******
DRB1*1327       --------EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ------I--D E--------- -----V---- -----H----
DRB1*1328       *******-EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E--------- ---R-V---* **********
DRB1*1329       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ---------D E--------- ---------- -----H----
DRB1*1330       ******--EY STS------- -----F-D-Y F-----Y--- ------F--- ------S--- ------I--D ---------- ---------- **********
DRB1*1331       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ------V--- ------I--D E--------- ---------- ----******
DRB1*1332       *****---EY STS------- -----F-D-Y FH----N--- ---------- ------S--- ------I--D E--------- -----V---- ----******
DRB1*1333       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D K-----N--- ------**** **********
DRB1*1334       *****---EY STS------- -----F-D-Y FH----NL-- ------F--- ---------- ------I--D E--------- ---------- -H--******
DRB1*1335       *****---EY STS------- -----F-D-Y FH----N--- ------FL-- ---------- ------I--D E--------- -----V---- ---*******
DRB1*1336       *****---EY STS------- -----F-D-Y FH----N--- ---------- ---------- ------I--D E--------- ---------- -----H----
DRB1*1337       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ---------- ------I--D K--------- ---------- ----******
DRB1*1338       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D E--------- ------**** **********
DRB1*1339       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------S ------I--D E--------- ---------- ----******
DRB1*1340       *******-EY STS------- -----F-D-Y FH----N--- ---------- ---------- ------I--D E--------- -----V---- ----******
DRB1*1341       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ------I--D E--------- ---------- ----******
DRB1*1342       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------F--D ---------- -----V---- ----******
DRB1*1343       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ------A--H ---------D E--------- -----V---- ----******
DRB1*1344       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ---------- ---------- ---------- -----V---- ----******
DRB1*1345       *******-EY STS------- -----F-D-Y F-----Y--- ------F--- ------A--H ------I--D E--------- -------*** **********
DRB1*1346       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ------V--- ------F--D ---------- ---------- ----******
DRB1*1347       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ---------- ------F--D ---L------ ---------- ----******
DRB1*1348       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D E--------- -----V---- ----******
DRB1*1349       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------F--D ---------- ---------- ---*******
DRB1*1350       *****---EY STS------- -----F-D-Y F-----N--- ------F--- ---------- ------F--D ---------- ---------- ----******
DRB1*1351       *****---EY STS------- -----F-D-Y FH----N--- ------F--L ---------- ------I--D E--------- -----V---- ----******
DRB1*1352       *****---EY STS------- -----F-D-Y FH----Y--- ------F--- ---------- ------I--D E--------- -----V---- -*********
DRB1*1353       *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ------I--D E--------- -----V---- ----******
DRB1*1354       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ------V--S ------F--D E--------- -----V---- ----******
DRB1*1355       ******--EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------F--D ---L------ ---------- ----******
DRB1*1356       --------EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ---------D ---------- ---------- -----H----
DRB1*1357       *****---EY STS------- -----F---Y FH----F--- ------F--- ---------- ------I--D E--------- -----V---- ----******
DRB1*1358       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D ---------- ----AV---- ----******
DRB1*1359       *****---EY STS------- -----FQD-Y FH----N--- ------F--- ---------- ------I--D E--------- -----V---- ----******
DRB1*1360       *****---EY STS------- -----F-D-Y F--------- ---------- ---------- ---------D ---------- ---------- ----******
DRB1*1361       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E-----N--- -----V---- ----******
DRB1*1362       *****---EY STS------- -----F-D-Y F-----DL-- ------F--- ---------- ------F--D ---------- ---------- ----******
DRB1*1363       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------F--D E--------- ---------- ----******
DRB1*1364       *****---EY STS------- -----F-D-Y FH----FL-- ------F--- ---------- ------I--D E--------- -----V---- ----******
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*1365       *****---EY STS------- -----F-D-Y FH----N--- ---------- ------S--- ------I--D E--------- ---------- ----******
DRB1*1366       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ------S--- ------I--D K--------- ---------- ----******
DRB1*1367       *****---EL --S------- -----F-D-Y FH----N--- ------F--- ---------- ------I--D E--------- ---------- ----******
DRB1*1368       *****---EY STS------- -----F-D-Y FH----D--- ------F--- ---------- ------I--D E--------- -----V---- ----******
DRB1*1369       *****---EY STS------- -----F-D-Y F-----N--- ------F--- ---------- ------I--D E--------- -----V---- ----******
DRB1*1370       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ---------- ------I--D E--------- -----V---- ----******
DRB1*1371       *****---EY STS------- -----Y-D-Y FH----N--- ------F--- ---------- ---------D E--------- -----V---- ----******
DRB1*1372       *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ------I--D E--------- -----V---- ----******
DRB1*1373       *****---EY STS------- ----LF-D-Y FH----N--- ------F--- ---------- ------I--D E--------- ---------- ----******
DRB1*1374       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ------IR-D E--------- ---------- ----******
DRB1*1375       *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ------S--- ------F--D E--------- -----V---- ----******
DRB1*1376       *****---EY STS------- -----F-D-Y FH----F--- ---------- ---------- ------I--D E--E------ -----V---- ----******
DRB1*1377       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ------V--S ------I--D ---------- -----V---- ----******
DRB1*1378       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------S ---------D E--------- -----V---- ----******
DRB1*1379       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------S ------I--D E--------- -----V---- ----******
DRB1*1380       *****---EY STS------- -----F-D-Y FH----N--- ------FW-- ---------- ------I--D E--------- -----V---- ----******
DRB1*1381       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------S--- ------I--D K--------- ----AV---- ----******
DRB1*140101     --------EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----V---- -----H----
DRB1*140102     *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----V**** **********
DRB1*140103     *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----V---- ----******
DRB1*1402       --------EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- ---------- ---------- -----H----
DRB1*140301     --------EY STS------- -----F---Y FH----N--- ---------- ---------- ---------D ---L------ ---------- -----H----
DRB1*140302     *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ---------D ---L------ ---------- ----******
DRB1*1404       --------EY STG--Y---- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----V---- -----H----
DRB1*140501     --------EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ---------R ---E------ -----V---- -----H----
DRB1*140502     *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ---------R ---E------ -----V---- ----******
DRB1*140503     *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ---------R ---E------ -----V---- ----******
DRB1*1406       *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- ---------- -----V---- -----H----
DRB1*140701     *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ ---------- -----H----
DRB1*140702     *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ ---------- ----******
DRB1*1408       *****---EY STS------- -----F-D-Y FH----F--- ---------- ---------H ---------R ---E------ -----V---- ----******
DRB1*1409       ******--EY STS------- -----F-D-Y FH----N--- ---------- ---------- ---------- ---------- ---------- ----******
DRB1*1410       *****---E- V-H------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----V---- -----H----
DRB1*1411       --------EY STG--Y---- -----F-D-Y FH----F--- ---------- -------E-- ---------R ---E------ -----V---- -----H----
DRB1*1412       *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ---------D ---L------ -----V---- ----******
DRB1*1413       *****---EY STS------- -----F---Y FH----N--- ---------- ------S--- ---------- ---------- ---------- -----H----
DRB1*1414       *****---EY STS------- -----F-D-Y FH----F--- ---------- ---------- ---------R ---E------ ---------- -----H----
DRB1*1415       *****---EY STG--Y---- -----F-D-Y FH----F--- ---------- ---------- ------F--D ---L------ -----V---- ----******
DRB1*1416       *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ------I--D E--------- -----V---- ----******
DRB1*1417       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ---------- ---------- -----V---- ----******
DRB1*1418       *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ---------R ---E------ -----V---- ----******
DRB1*1419       --------EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- K--------- ---------- **********
DRB1*1420       *******-EY STS------- -----F---Y FH----F--- ---------- ---------- ---------- ---------- -----V-*** **********
DRB1*1421       *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ---------- K--------- -----V---- -----H----
DRB1*1422       *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ------F--D ---------- ---------- ----******
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*142301      *****---EY STS------- -----F-D-Y FH----F--- ---------- ---------- ---------R ---E------ -----V---- ----******
DRB1*142302      *****---EY STS------- -----F-D-Y FH----F--- ---------- ---------- ---------R ---E------ -----V---- ----******
DRB1*1424        ******--EY STS------- -----F---Y FH----N--- ---------- ---------- ------I--- A--------- ---------- -----H----
DRB1*1425        --------EY STS------- -----F-D-Y F-----Y--- ---------- ------A--H ------F--D ---------- ---------- ----******
DRB1*1426        *****---EY STS------- ----QF-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----V---- ----******
DRB1*1427        *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ------F--D ---L------ ---------- ----******
DRB1*1428        *****---EY STG--Y---- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ ----AV**** **********
DRB1*1429        *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- ----AV---- ----******
DRB1*1430        *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ---------- ---------- ----******
DRB1*1431        ******--EY STG--Y---- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---------- -----V---- **********
DRB1*143201      *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---------- -----V---- ----******
DRB1*143202      *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---------- -----V---- ----******
DRB1*1433        *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------- ---------- ---E------ -----V---- ----******
DRB1*1434        *****---EY STS------- -----F-D-Y FH----F--- ---------- ---------H ---------R ---------- -----V---- ----******
DRB1*1435        *****---EY STS------- -----F-D-Y FH----F--- ------F--- ------A--H ---------R ---E------ -----V---- ---*******
DRB1*1436        *****---EY STS------- -----F-D-Y FH----F--- ----R----- ---------- ---------R ---E------ ---------- ----******
DRB1*1437        *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ------I--- A--------- -----V---- ----******
DRB1*1438        *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E--N--- -----V---- ----******
DRB1*1439        *****---EY PTS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----V---- ----******
DRB1*1440        *****---EY STS------- -----F---Y FH----F--- ---------- ---------- ---------D ---L------ ---------- ----******
DRB1*1441        *****---EY STS------- -----F---Y FH----FL-- ---------- ---------- ---------- ---------- ---------- ----******
DRB1*1442        *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ---------- ---------R ---E------ ---------- ----******
DRB1*1443        *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ---------R ---E--A--- -----V---- ----******
DRB1*144401      *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ---------R ---E------ ---------- ----******
DRB1*144402      *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ---------R ---E------ ---------- ----******
DRB1*1445        *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ------I--R ---E------ -----V---- ----******
DRB1*1446        --------EL R-S------- -----F---Y FH----N--- ---------- ---------- ---------- ---------- ---------- -----H----
DRB1*1447        *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- ------N--- ---------- ----******
DRB1*1448        *****---EY STS------- -----F---Y FH----N--- ---------- ------V--S ---------- ---------- ---------- ----******
DRB1*1449        *****---EY STS------- -----F---Y FH----F--- ---------- ------A--H ---------- ---------- ---------- ----******
DRB1*1450        *****---EY STG--Y---- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E--N--- -----V---- ----******
DRB1*1451        *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ---------- ---E------ ---------- ----******
DRB1*1452        *****---EY STG--Y---- -----F---Y FH----N--- ---------- ---------- ---------- ---------- -----V---- ----******
DRB1*1453        *****---EY STS------- -----F-D-Y F-----Y--- ------F--- ------A--H ------F--D ---------- ---------- ----******
DRB1*1454        *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----V---- -----H----
DRB1*1455        *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---L------ -----V---- ----******
DRB1*1456        *****---EY STS--Q---- -----F-D-Y FH----F--- ---------- ---------- ---------R --SE------ -----V---- ----******
DRB1*1457        --------E- V-H------- -----F-D-Y FH----F--- ---------- ------A--- ------I--D E--------- -----V---- -----H----
DRB1*1458        *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------A--H ---------R ---E------ -----V---- ----******
DRB1*1459        *****---EY STS------- -----F-D-Y FH----N--- ---------- R--------- ---------R ---E------ -----V---- ----******
DRB1*1460        *****---EY STS------- -----F-D-Y FH----F--- ----R----- ------A--H ---------R ---E------ -----V---- ----******
DRB1*1461        *****---EY STG------- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----V---- ----******
DRB1*1462        *****---EY STS------- -----F-D-Y FH----F--- ---------- ------T--H ---------R ---E------ -----V---- ----******
DRB1*1463        *****---EY STS------- -----F---Y FH----N--- ---------- ------S--- ------I--D ---L------ ---------- ----******
DRB1*1464        *****---EY STS------- -----F-D-Y FH----N--- ------F--- ---------S ---------R ---E------ -----V---- ----******
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*1465       *****---EY STS------- -----F-D-Y FH----F--- ------F--- ------S--- ---------R ---------- -----V---- ----******
DRB1*1467       *****---EY STS------- -----F---Y FH----N--- ---------- ---------- ------I--D ---L------ ---------- ----******
DRB1*1468       *****---EY STG--Y---- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ ---------- ----******
DRB1*1469       *****---EY STS------- -----F-D-Y F-----Y--- ---------- ------A--H ---------D ---------- ---------- ----******
DRB1*1470       *****---EY STS------- -----F-D-H FH----F--- ---------- ------A--H ---------R ---E------ -----V---- ----******
DRB1*1471       *****---EY STG--Y---- -----F-D-Y FH----F--- ---------- ------A--H ---------R ---E------ -----M---- ----******
DRB1*1472       *****---EY STS------- -----F-D-Y FH----F--- ------F--- ---------H ---------R ---E------ -----V---- ----******
DRB1*1473       *****---EY STG--Y---- -----F-D-Y FH----F--- ---------- ------A--H ------F--D ---E------ -----V---- ----******
DRB1*1474       *****---EY STS------- -----F-D-Y FH----F--- ---------- ------A--H ---------D ---------- -----V---- ----******
DRB1*150101     ---------- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- -----V---- -----Q----
DRB1*150102     *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- -----V---- ---*******
DRB1*150103     *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- -----V---- ---*******
DRB1*150104     *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- -----V---- ----******
DRB1*150105     *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- -----V---- ----******
DRB1*150106     *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- -----V---- ----******
DRB1*150201     ---------- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- ---------- -----Q----
DRB1*150202     *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- ---------- -----Q----
DRB1*150203     *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- ------**** **********
DRB1*150204     *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- ---------- ----******
DRB1*1503       ---------- P-R------- -----F-D-H F--------- ------F--- ---------- ------I--- A--------- -----V---- -----Q----
DRB1*1504       ---------- P-R------- -----F-D-Y F--------- ------F--- ---------- ------F--- A--------- -----V---- -----Q----
DRB1*1505       ******---- P-R------- -----F-D-Y F--------- ------F--- ---------- ---------- A--------- -----V---- ---*******
DRB1*1506       ********-- P-R------- -----F-D-Y F--------- ------F--A ---------- ------I--- A--------- -----V---- ----******
DRB1*1507       *****----- P-R------- -----F-D-Y F--------- ---------- ---------- ------I--- A--------- -----V---- -----Q----
DRB1*1508       *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- -----NI--- A--------- ---------- ----******
DRB1*1509       *****----- P-R------- -----F-D-Y F--------- ------FQ-- ---------- ------I--- A--------- -----V---- ----******
DRB1*1510       *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--D E--------- -----V---* **********
DRB1*1511       *****----- P-R------- -----F-D-Y F--------- ---------- ---------- ------I--- A--------- ---------- ----******
DRB1*1512       *****----- P-R------- -----F-D-Y F--------- ------F--- ------S--- ------I--- A--------- -----V---- ----******
DRB1*1513       *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ----.-I--- A--------- -----V---- ---*******
DRB1*1514       *****----- P-R------A -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- ---------- ----******
DRB1*1515       *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------F--- A--------- ---------- ----******
DRB1*1516       *****----- P-R------- -----F-D-Y F--------- ----R-F--- ---------- ------I--- A--------- -----V---- ----******
DRB1*1517N      *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--D --GPRW-PTA DTTTGLWRAS QCSGESNLRX
DRB1*1518       *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--E A--------- -----V---- ----******
DRB1*1519       *****G---- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- ---------- ----******
DRB1*1520       *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--- A--------- ---R-V---- ----******
DRB1*1521       *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------I--D ---L------ -----V---- ----******
DRB1*1522       *****----- P-R------- -----F-D-Y F--------- ------F--- ----L----- ------I--- A--------- -----V---- ----******
DRB1*1523       *****----- P-R------- -----F-D-H F--------- ------F--- ---------- ---------- A--------- -----V---- ----******
DRB1*1524       *****----- P-R------- ----QF-D-Y F--------- ------F--- ---------- ------I--- A--------- -----V---- ----******
DRB1*1525       *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ---------- K-GR--N--- -----V---- ----******
DRB1*1526       *****----- P-R------- S----F-D-Y F--------- ------F--- ---------- ------I--- A--------- ---------- ----******
DRB1*1527       *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ---------- ---------- ---------- ----******
DRB1*160101     ---------- P-R------- -----F-D-Y F--------- ---------- ---------- ------F--D ---------- ---------- -----Q----
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Prot. Pos.              10         20         30         40         50         60         70         80         90        100
DRB1*160102      *****----- P-R------- -----F-D-Y F--------- ---------- ---------- ------F--D ---------- ---------- **********
DRB1*160201      ---------- P-R------- -----F-D-Y F--------- ---------- ---------- ---------D ---------- ---------- -----Q----
DRB1*160202      ******---- P-R------- -----F-D-Y F--------- ---------- ---------- ---------D ---------- ------**** **********
DRB1*1603        ---------- P-R------- -----F-D-Y F--------- ---------- ---------- ------F--D -A-------- ---------- -----Q----
DRB1*1604        *****----- P-R------- -----F-D-Y F--------- ---------- ---------- ------F--D ---L------ ---------- -----Q----
DRB1*160501      *******--- P-R------- -----F-D-Y F--------- ---------- ---------- ------I--D ---------- -------*** **********
DRB1*160502      *****----- P-R------- -----F-D-Y F--------- ---------- ---------- ------I--D ---------- ---------- ----******
DRB1*1607        *****----- P-R------- -----FPD-Y F--------- ---------- ---------- ------I--D ---------- ---------- -*********
DRB1*1608        *****----- P-R------- -----F-D-Y F-----N--- ---------- ---------- ------F--D ---------- ---------- ----******
DRB1*1609        *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ------F--D ---------- ---------- ----******
DRB1*1610        *****----- P-R------- -----F-D-Y F--------- ------F--- ---------- ---------D ---------- ---------- ----******
DRB1*1611        *****----- P-RK------ -----F-D-Y F--------- ---------- ---------- ---------D ---------- ---------- ----******
DRB1*1612        *****----- P-K------- -----F-D-Y F--------- ---------- ---------- ---------D ---------- ---------- ----******
DRB1*1613N       *****----- P-R------- -----F-D-Y F--------- ---------- -X
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Appendix 2 Hyperladder IV DNA quantification

HyperLadder IV (Bioline, UK) can be used for DNA quantification based on band brightness. The following quantities of DNA are found in bands of varying volumes of HyperLadder IV. Information taken from Bioline website [213]

	Volume of HyperLadder IV µl **DNA (ng)**				
Band (bp)	**1µl**	**2µl**	**3µl**	**4µl**	**5µl**
1000	20	40	60	80	100
900	18	36	54	72	90
800	16	32	48	64	80
700	14	28	42	56	70
600	12	24	36	48	60
500	10	20	30	40	50
400	8	16	24	32	40
300	6	12	18	24	30
200	4	8	12	16	20
100	8	16	24	32	40

Appendix 3 Amino Acid variations between HLA-DR alleles used in this study

Sequences are taken from the IMGT database [39]. Key residues for SAg binding identified by crystallographic studies are highlighted in green.

	Amino Acid position																				
***DRB1* allele**	**66**	**67**	**68**	**69**	**70**	**71**	**72**	**73**	**74**	**75**	**76**	**77**	**78**	**79**	**80**	**81**	**82**	**83**	**84**	**85**	**86**
Group 1																					
**0403*	D	L	L	E	Q	R	R	A	A	V	D	T	Y	C	R	H	N	Y	G	V	V
**0404*	D	L	L	E	Q	R	R	A	A	V	D	T	Y	C	R	H	N	Y	G	V	V
Group 2																					
**0701*	D	I	L	E	D	R	R	G	Q	V	D	T	V	C	R	H	N	G	Q	V	G
**1101*	D	F	L	E	D	R	R	A	A	V	D	T	Y	C	R	H	N	Y	G	V	G
**1301*	D	I	L	E	D	E	R	A	A	V	D	T	Y	C	R	H	N	Y	G	V	V