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Functional Characterisation of Superantigens in *Staphylococcus aureus* Disease Pathogenesis

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Declaration

I declare that I have composed this thesis and the work described here is my own. It is clearly indicated where the work described was performed by collaborating researchers. This work has not been submitted for any other degree or professional qualification.

Stephen Nutbeam-Tuffs

Dedication

I would like to dedicate this work to the memory of Derrick Weeks and Moira Martin

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LIST OF COMMONLY USED ABBREVIATIONS

Abbreviation	Full description
α	alpha
agr	accessory gene regulator
AIP	auto inducing peptide
APC	antigen presenting cell
APS	ammonium persulphate
β	beta
BLAST	basic local alignment search tool
bp	base pair
BHI	brain heart infusion
°C	degrees Celsius
CA-MRSA	Community associated methicillin-resistant *Staphylococcus aureus*
CC	clonal complex
CFU	colony forming units
CD	circular dichroism
CD-	cluster of differentiation
CO_2	carbon dioxide
cpm	counts per minute
C_T	cycle threshold
CWA	cell wall associated
d	days
ddH_2O	double distilled water
DMSO	dimethyl sulpoxide

Abbreviation	Full description
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	ethylene-diamine-tetra acetic acid
egc	enterotoxin gene cluster
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
x g	force of gravity
γ	gamma
g	grams
gDNA	genomic DNA
h	hours
HA-MRSA	Hospital-associated methicillin-resistant *Staphylococcus aureus*
HBSS	hanks balanced salt solution
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGT	horizontal gene transfer
^{3}H	hydrogen radioactive isotope 3 (tritium)
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interlukin
IPTG	β-D-1-thiogalactpyranoside
IMAC	immobilised metal affinity chromatography
kDa	kilo-Dalton

Abbreviation	Full description
kV	kilo-volts
LA-MRSA	Livestock-associated methicillin-resistant *Staphylococcus aureus*
L	litres
LB	Luria broth
LC-MS/MS	liquid chromatography tandem mass spectrometry
kb	kilo-base
MEGA	Molecular Evolutionary Genetics Analysis
mM	milli-molar
MGE	mobile genetic element
MHC	major histocompatibility complex
min	minutes
µCi	micro-curie
µg	micro-grams
µF	micro-Faraday
µl	micro-litres
ml	milli-litres
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MWCO	molecular weight cut off
NCBI	National Centre for Biotechnology Information
ng	nano-grams
nm	nano-meter
nM	nano-molar

Abbreviation	Full description
NK	not known
NTC	no template control
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDB	protein data bank
PE	phycoerythrin
PMN	polymorphonuclear leukocytes
PSG	penicillin, streptomycin, L-glutamine
PSGL-1	P-selectin glycoprotein ligand-1
PVL	Panton-Valentin leukocidin
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
RBC	red blood cells
RNA	ribonucleic acid
Rot	repressor of toxins
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	reverse transcriptase
s	second
SAB	sialic acid binding motif
SAg	superantigen
sae	*Staphylococcus aureus* exoprotein regulator
SaPI	staphylococcal pathogenicity islands
SDS	sodium dodecyl sulphate

Abbreviation	Full description
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SE	staphylococcal enterotoxin
SEl	staphylococcal enterotoxin-like
Src	sarcoma
ST	sequence type
TAE	tris-acetate ethylene diamine-tetra-acetic acid
TCR	T-cell receptor
TCS	two component system
TE	Tris-ethylene-diamine-tetra-acetic acid
TEMED	tetramethylethylenediamine
T_h1	T helper 1
T_h2	T helper 2
T_h17	T helper 17
TNF	Tumour necrosis factor
T_{regs}	Regulatory T-cells
TSA	tryptone soya agar
TSB	tryptone soya broth
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin-1
U	units
UV	ultraviolet
V	volts
v/v	volume for volume
WT	wild type
w/v	weight for volume

ABSTRACT

Bacterial superantigens (SAgs) are virulence factors that induce nonspecific T-cell proliferation contributing to host immune avoidance, and occasionally severe life-threatening toxinoses such as toxic shock syndrome. In the current study, the multiple functions of 3 superantigens named staphylococcal enterotoxin-like toxins X, Y and Z are investigated. SElX and SElZ were non-emetic in a musk shrew model of emesis. SElX is structurally and phylogenetically related to staphylococcal superantigen-like proteins (SSls) which are non-mitogenic but exhibit a variety of immune modulatory properties. We carried out protein and gene expression analysis of mutants of different *S. aureus* gene regulators and demonstrated that *selx* expression is controlled by *saeRS*, a two-component regulator linked to the bacterial response to phagocytic signals. Considering the co-regulation of SElX with known mediators of innate immune evasion we investigated a potential role for SElX in both humoral and cellular innate immune modulation and discovered that SElX strongly binds to human, bovine, murine, and laprine neutrophils and interferes with IgG-mediated phagocytosis, independently of Fcγ receptor signalling. Bacterial survival assays with neutrophils demonstrated that the deletion of *selx* significantly reduced the ability of *S. aureus* to resist neutrophil killing. Site-directed mutagenesis in the conserved sialic acid-binding motif of SElX abolished its neutrophil binding capacity, which is consistent with a critical role for glycosylated receptors in this interaction. Importantly, the sialic-acid binding mutants of SElX retained the ability to induce T-cell proliferation demonstrating that the distinct functions of SElX are mechanistically independent. Affinity precipitation experiments identified potential glycoprotein receptors for SElX and the interaction with protein ICAM-3, an important ligand for MAC-1 integrins, was validated suggesting SElX may interfere with cell signalling. Taken together, we present the first example of a bi-functional SAg that can manipulate two distinct arms of the human immune system and contribute to *S. aureus* survival during infection.

LAY SUMMARY

The bacterium *Staphylococcus aureus* is responsible for an array of diseases in both humans and animals. *S. aureus* infection can cause serious life threatening illness in humans in both the hospital and community setting. *S. aureus* produces a range of virulence factors that allow the bacteria to establish and maintain an infection by mediating transmission, entry into the host and avoidance of the immune system. In the current study the *S. aureus* proteins SElX, SElY and SElZ were examined to determine how these factors are deployed by *S. aureus* to contribute to infection. The three proteins examined have already been shown to be superantigens, which are proteins that induce non-specific T-cell activation. T-cells are important signalling cells in the immune system and by activating T-cells non-specifically can cause dysregulation of the immune system. SElY and SElZ have been recently identified and so far been poorly characterised, in the current study the mechanisms of superantigenicity of these proteins has been better defined. The superantigens are a diverse group of proteins and may exhibit a range of different functions. In this study we demonstrated that SElY is highly resistant to variations in temperature and pH which may contribute to its role as a food poisoning toxin. We also demonstrated that the protein SElX can inhibit the function of a group of immune cells called neutrophils. These cells are important in clearing *S. aureus* infections as they take up the bacteria and kill them. If these cells are being inhibited by SElX it can contribute further to immune avoidance by *S. aureus*. This is the first example of a superantigen exhibiting more than one immune evasion function. Within the field of *S. aureus* disease the observation of *S. aureus* virulence factors exhibiting more than one function is increasing. Understanding the full functional complement of virulence factors secreted by *S.aureus* is important as it will allow the development of targeted therapies that can block all functions of *S. aureus* virulence factors and prevent immune avoidance by the bacteria that leads to treatment failure.

Chapter 1

Introduction

1.1 *Staphylococcus aureus*

1.1.1 Microbiology

Staphylococcus aureus is a facultatively anaerobic Gram-positive coccal bacterium that is responsible for an array of diseases in both the hospital and the community setting. *S. aureus* is a commensal commonly found in the anterior nares of humans with colonisation rates of 25% to 30% of healthy individuals and can also be isolated from the axillae, vagina, pharynx or damaged skin (Thomas et al., 2007, Dinges et al., 2000, Wilson et al., 2011). Under certain circumstances *S. aureus* can deploy a wide variety of virulence determinants which can lead to local and systemic diseases in humans including sepsis, necrotizing pneumonia, and toxic shock syndrome (TSS) (Choi et al., 1989). *S. aureus* has also been implicated in an array of veterinary diseases including blood stream infections, mastitis and pneumonia in a number of hosts including horses, cattle and poultry (Dellabona et al., 1990, Drake and Kotzin, 1992).

1.1.2 *S. aureus* infections; clinical presentation and epidemiology

S. aureus is a common cause of infection in the hospital environment associated with a number of clinical complications including skin and soft tissue infections (SSTIs), bloodstream infections (BSIs) and ventilator-associated pneumonia (VAP) (Fraser and Proft, 2008a, Kawabe and Ochi, 1990, Ferens and Bohach, 2000). Methicillin resistant *Staphylococcus aureus* (MRSA) is widespread in hospitals across the world (Sol et al., 2000), and is estimated to cause 171 200 healthcare-associated infections in Europe each year (Thomas et al., 2009). MRSA represents a serious clinical problem owing to the varied level of antibiotic resistance found in different strains of this organism (Seo et al., 2010b, Ono et al., 2008b).

SSTI with *S. aureus* can range in severity and can present in many forms including abscesses, impetigo, cellulitis and necrotising fasciitis (Deringer et al., 1997). *S. aureus* was already the leading cause of SSTIs worldwide but the incidence has increased dramatically since the emergence of community associated MRSA (CA-MRSA) (Deringer et al., 1996). The most common *S. aureus* SSTIs presented in the clinic are cutaneous abscesses which normally can be resolved by drainage and antibiotic treatment (Fitzgerald et al., 2001a). Skin infections can also result from 2 % to 4 % of surgical procedures, with *S. aureus* found to be the most common cause of infections, accounting for 30 % of cases (Connelley et al., 2009, Elsik et al., 2009). Other manifestations of SSTIs include impetigo, which is the most common form of skin infection in children, and *S. aureus* is the most common pathogen isolated from these infections (70 % of cases) (Wilson et al., 2011). Necrotizing fasciitis is another condition in which *S. aureus* can play a role; it is associated with patients that have *S. aureus*-infected wounds in addition to other medical complications such as diabetes or hepatitis C (Herron-Olson et al., 2007b). In a hospital study in Taiwan 38% of patients presenting with necrotising fasciitis were found to be culture-positive for *S. aureus* and 60% of these isolates were found to be MRSA (Wilson et al., 2011). This demonstrates that *S. aureus* remains a serious clinical problem both in the hospital and in the community.

S. aureus is responsible for other life-threatening diseases including infective endocarditis (IE) and is the leading cause of this condition worldwide (Rozen, 2000, Enright et al., 2000). In the USA the increasing incidence of IE has been attributed to a higher incidence of *S. aureus*, and IE rates have increased by 27 % (1998 to 2009) mirrored by a 25 % increase in *S. aureus*-associated IE cases (Tamura et al., 2007). IE occurs when the endocardium is damaged which can result from catheters, turbulent blood flow from vascular abnormalities or particulates from intravenous drug use (Ster et al., 2005). This damage results in the deposition of platelets and fibrin from the exposure of the subendothelial cells which leads to the formation of sterile vegetations (Wilson et al., 2011). These vegetations

can become infected and *S. aureus* is highly suited for this as it encodes a number of proteins that bind to components in the vegetation (Goddeeris and Morrison, 1988, Wilson et al., 2011). The clinical consequences of this infection include severe cardiac damage and dysfunction, persistent fever and peripheral emboli (Wilson et al., 2011, Bremel, 1980).

The formation of IE often occurs after the development of *S. aureus* bacteraemia which has an incidence of 10 % to 30 % per 100,000 persons-years in developed nations (Ward and Schultz, 1973). There are a number of factors which pre-dispose patients to *S. aureus* bacteraemia including human immunodeficiency virus (HIV) infection, intravenous drug use and hospital procedures such as haemodialysis (Wilson et al., 2011). SSTI infection can also progress to bacteraemia and the incidence of this has increased along with the incidence of CA-MRSA SSTIs (Luong et al., 2002).

S. aureus has been shown to be one of the leading causes of nosocomial pneumonia associated with 28 % to 40 % of infections in the USA (Herron-Olson et al., 2007b, Ono et al., 2008b). Incidence of *S. aureus* community associated pneumonia (CAP) is relatively low (2 % to 3 % of cases) (Lina et al., 2004a). However *S. aureus* CAP is associated with poorer outcomes for the patient, particularly when the disease manifests as necrotising pneumonia (Thomas et al., 2007). The emergence of necrotising pneumonia is associated with a number of *S. aureus* isolates including clinically-important strains such as the USA300 CA-MRSA clone (Orwin et al., 2002, Thomas et al., 2007, Lina G., 2004). The pathology observed in *S. aureus* pneumonia is primarily mediated by the hosts own immune response; it is characterised by a large leukocyte infiltration resulting in severe tissue damage from inflammation, and the activity of a number of toxins secreted by *S. aureus* including α-haemolysin (Wilson et al., 2011, Fitzgerald et al., 2001a, Bramley et al., 1989). Mortality resulting from nosocomial *S. aureus* pneumonia can be as high as 50 % and mortality rates for *S. aureus* CAP were found to be equally high at 56 % (Somerville et al., 2002, Vojtov et al., 2002).

1.1.3 Community-associated methicillin resistant *S. aureus* (CA-MRSA)

CA-MRSA was first identified in Australia in 1993, and has become a serious clinical problem worldwide (Fitzgerald et al., 2001a, Deringer et al., 1997). In the last 30 years since emerging, CA-MRSA has established itself and diversified in the human population (Deringer et al., 1996). CA-MRSA tends to affect patients who are seen as healthy and have no risk factors associated with MRSA. In contrast to HA-MRSA, people infected by these strains tend to be younger, with median age for CA-MRSA of 23 years versus 68 years median age for HA-MRSA (Wilson et al., 2011).

The pulse field gel electrophoresis (PFGE) CA-MRSA clone USA300 has become dominant in the USA (Fitzgerald et al., 2001a, Deringer et al., 1997, Wilson et al., 2011) and is the leading clone of *S. aureus* associated with SSTIs in the USA (Choi et al., 1989, Connelley et al., 2009, Elsik et al., 2009). USA300 isolates emerged from the multilocus sequence type (MLST) clonal complex (CC) 8, by acquisition of the staphylococcal chromosome cassette *mec* (SCC*mec*) type IV and are typically characterised by the presence of the Panton-Valentin leucocidin (PVL) gene (Tenover et al., 2006). The role of this toxin in *S. aureus* pathogenesis will be discussed in more detail in section 1.5.3.

USA300 is the causative agent of a wide array of life threatening diseases including necrotising pneumonia, severe sepsis and necrotising fasciitis (Holtfreter et al., 2007). It has occurred in a variety of community settings including new-born nurseries, military barracks and amongst athletics teams (Guinane et al., Lowder et al., 2009a, Herron-Olson et al., 2007a). It has been reported that USA300 isolates can spread within individual households and inferred to persist between 2-8 years (Smyth et al., 2009). This can help explain why some patients, who are susceptible to SSTI caused by USA300, keep suffering recurrent infections (Fitzgerald et al., 2001a). There is evidence to suggest that unlike other clinical *S. aureus* strains, the USA300 clone relies more on core-genome encoded virulence factors and

differential regulation of these factors rather than determinants encoded in the accessory genome (Gaskill and Khan, 1988, Tseng et al., 2004).

Also associated with USA300 isolates is the carriage of the arginine catabolic mobile element (ACME) which may contribute to the success of USA300 (Regassa et al., 1991). ACME in USA300 has been shown to have a very high level of homology to an element found in *S. epidermidis* and it is hypothesised that the transfer of ACME occurred from *S. epidermidis* into the USA300 lineage around the time the ancestor of this clone emerged (Derzelle et al., 2009, Guinane et al., 2008). The role of ACME in *S. aureus* pathogenesis has not been clearly defined but it has been reported the element encodes SpeG, an enzyme linked to the degradation of polyamines which are secreted by the skin and are toxic to *S. aureus* (Herron-Olson et al., 2007a, Shaw et al., 2006).

1.2 Antibiotic resistance in *S. aureus*

Methicillin resistance is mediated by the SCC*mec* element which contains *mecA* encoding penicillin binding protein 2A (PBP2A) which confers resistance to all β-lactam antibiotics (Guinane et al., 2008). Analysis of the *S. aureus* sequence type (ST) 5 demonstrates that this clone appeared to have evolved multiple times, in many countries, through acquisition of the *SCCmec* element by methicillin sensitive strains (Nubel et al., 2008). This suggests that methicillin resistance arises locally rather than spread over a geographical area of a single transformed clone (Kazmi et al., 2001, Broudy et al., 2001). SCC*mec* has diversified quite considerably, with at least 10 SCC*mec* types characterised to date (Kazmi et al., 2001, Broudy et al., 2001). As a result SCC*mec* can vary greatly in size and the type of resistance types of antibiotics other than β-lactams that they can confer resistance to. For example, the SCC*mec* type II can encode resistance to erythromycin, spectinomycin and tobramycin (Takei et al., 1993).

Vancomycin has been the gold standard antibiotic for managing serious MRSA infection for some time but due to its widespread use strains have emerged with decreased susceptibility (Ulrich, 2000, Holtfreter et al., 2004). These strains, termed vancomycin intermediate *Staphylococcus aureus* (VISA), have a minimum inhibitory concentration (MIC) above 2 mg/l (Holtfreter et al., 2004). Strains with MIC of more than 2 mg/l are associated with a much higher level of treatment failure for patients with severe MRSA infection (Wilson et al., 2011). The prevalence of these strains has increased from 2.6 % in 2004 to 5.6 % in 2009 (Fitzgerald et al., 2001a, Jarraud et al., 2001a). High level vancomycin resistance can be conferred by the transfer of the VanA plasmid from *Enterococcus* species, although this is still rare (Johns and Khan, 1988, Fitzgerald et al., 2001a). New antimicrobials have been tested and shown promise in the clinic including daptomycin (Jarraud et al., 2001a). These new compounds have also been developed alongside the deployment of new therapy strategies which include the use of combined administration of β-lactams with vancomycin or daptomycin and have been shown to improve patient outcomes for serious skin infections and pneumonia (Ono et al., 2008b, Johns and Khan, 1988). While novel antibiotics and strategies are being developed, the continued increase of antimicrobial resistance constantly threatens the efficacy of these treatments.

1.3 *S. aureus* Evolution and Genetics

1.3.1 Genome organisation of *S. aureus*

Evolution of *S. aureus* has led to the generation of a diverse group of strains which can colonise a wide range of hosts, evade the immune system of these hosts and deploy an array of strategies to resist antibiotics (Lindsay and Holden, 2006, Ben Zakour et al., 2008). The genome of *S. aureus* can be divided into a core-, core variable- and an accessory region

(Fernandez et al., 2006). The core genome of *S. aureus* is composed of approximately 75 % of genes and includes all genes essential for growth, survival and some virulence and resistance genes found in all strains of *S. aureus* (Jarraud et al., 2001a, Thomas et al., 2006). The core variable component, making up approximately 10% of the *S. aureus* genome, is composed of genes shared by strains of the same evolutionary lineage which may exhibit a higher rate of nucleotide substitution compared to core genes (Ho et al., 1989, Deringer et al., 1997). The core variable component often includes genes associated with niche adaption and host species specificity in colonisation and infection (Marr et al., 1993).

The accessory genome is composed of genes that vary from strain to strain and contribute to non-essential functions such as antimicrobial resistance, alternative metabolic substrate utilization and virulence determinants (Marr et al., 1993). The accessory genome of *S. aureus* is composed of mobile genetic elements (MGE) such as prophages, plasmids and genomic islands including the *S. aureus* pathogenicity islands (SaPIs) (Fitzgerald et al., 2001a). MGEs allow *S. aureus* to rapidly adapt to new niches and respond to new selective pressures such as antibiotics (Wilson et al., 2011, Lina et al., 2004b). MGEs can be transferred through three major mechanisms: transformation (the uptake of naked DNA), transduction (bacteriophage mediated transfer) and conjugation (exchange of DNA through direct contact) (Lina et al., 2004b).

1.3.2 Mechanisms of *S. aureus* evolution

In *S. aureus*, point mutations have been show to outnumber recombination events leading to a clonal population structure (Ono et al., 2008b, Seo et al.). The generation time of bacteria allows successful alleles to become dominant relatively quickly throughout a clonal population (Thomas et al., 2009, Ono et al., 2008a). The non-synonymous mutations generated through this mechanism can help contribute to host adaption. For example, single

nucleotide polymorphisms (SNPs) in the *dltB* gene of *S. aureus* have been shown to allow human strains to adapt to the rabbit host in parallel in multiple lineages (Seo et al.). Point mutations have also been shown to contribute to the resistance of *S. aureus* to certain antibiotics including daptomycin, vancomycin and rifampicin (Thomas et al., 2009).

Bacteriophages play a major role in disseminating genes across the *S. aureus* population and there are many examples of the dissemination of important virulence factors transmitted by the activity of phage in *S. aureus* (Ono et al., 2008a, Deringer et al., 1997). The genes *LukSF* which encode the leukocidin PVL have been shown to be transferred through *S. aureus* populations by a diverse group of bacteriophages (Fitzgerald et al., 2001a). The β-haemolysin converting phage carries the immune evasion cluster (IEC) which inserts into the β-haemolysin gene encoding the virulence genes *scn, chp, sak* and *sea* (Fitzgerald et al., 2001a). The acquisition of genes from phage can allow *S. aureus* to adapt to a new host as exemplified by ruminant adapted SaPIs (e.g. SaPIov2), which are transmitted by phage and can transmit host adapted alleles of important virulence factors such as the ruminant-specific von Willebrand factor binding protein (vWBP) (Thomas et al., 2009).

Plasmid-encoded antibiotic resistance has been well reported in *S. aureus* and these plasmids have been shown to be able to confer resistance to antimicrobials across many *S. aureus* isolates (Thomas et al., 2009). Plasmids have been shown to carry multiple resistance genes and virulence determinants in *S.aureus*. The plasmid-encoding exfoliative toxin B plasmid, pETB, has also been reported to carry multiple resistance genes which confer resistance to penicillin, erythromycin, aminoglycoside and gentamicin (Ferry et al., 2008). Other examples of plasmid-encoded virulence determinants include a number of superantigens which are found primarily on two plasmids, the pIB485-like plasmid (encoding *sej, ser* and *sed*) and the Pf5 plasmid (encoding *ser, sej, ses* and *set*) (Seo et al., Thomas et al., 2009, Connelley et al., 2009). *S. aureus* plasmids encoding the *crf* gene confer resistance to Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin

classes of antibiotics by methylation of the A2502 rRNA base in the 23S subunit of the *S. aureus* ribosome (Seo et al., 2010b). Carriage of plasmids encoding *crf* has been reported in a number of important clinical lineages of *S. aureus* including USA300 and the livestock associated MRSA lineage CC398 and there is evidence to suggest further dissemination of plasmids carrying this gene into other clinical isolates and into other species of staphylococci (Thomas et al., 2009, Deringer et al., 1997, Ebling et al., 2001, Kuroishi et al., 2003).

Large scale modifications of the *S. aureus* genome allowing for the evolution and adaption to new host species over time have been well reported (Ebling et al., 2001). However there has been recent evidence to suggest that *S. aureus* can exhibit a great deal of within-host diversity which can contribute to evolution and host adaption over the course of a single infection (Fitzgerald et al., 1997, Fitzgerald et al., 2000, Kuroishi et al., 2003). In a transmission network studied in a veterinary hospital the diversity of an *S. aureus* infection was examined and multiple isolates from a single canine host were sequenced (Dauwalder et al., 2009). This revealed that a number of SNPs had accumulated during the infection and non-synonymous changes were observed in genes including the *agr* quorum sensing gene regulator (Virtaneva et al., 2005). Lower activity of the *agr* regulator is associated with higher levels of antimicrobial resistance and indicates the bacterial population is responding to selective pressures in the host (Guinane et al., 2008, Tollersrud et al., 2006, Pellegrino et al., 2008).

Evolution and adaption to new host environments has been demonstrated to require both the gain and loss of genes (Tollersrud et al., 2006). It has been shown that the acquisition of the IEC is critical for the CC398 LA-MRSA isolates to be able to transfer and adapt to the human host (Guinane et al., 2008). Conversely isolates of CC8 from bovine hosts were found to be devoid of the β-haemolysin converting prophage (Anderson, 1982). This demonstrates that the accessory genome of *S. aureus* is highly dynamic, acquiring genes

that will aid survival in a new environment and losing redundant genes that may have a high fitness cost or generating a strong immune response in the host.

1.3.3 Restriction modification systems and other defences against foreign DNA

Restriction modification (RM) systems present a barrier to mobile elements but are necessary to prevent the destruction of a bacterial population by a lytic phage (Herron-Olson et al., 2007b). Type I RM systems are found in the majority of isolates of *S. aureus* and are made up of three subunits which form a complex that mediates the addition of a methyl group on to adenosine bases of target sequences and prevents the degradation of the sequence from restriction enzymes (Kenny et al., 1992, Bramley et al., 1989). The complex is made up of a restriction subunit (*hsdR*), a modification subunit (*hsdM*) and a specificity subunit (*hsdS*) (C.M. Guinane, 2007). The *hsdS* subunit is highly stable in single lineages and this allows DNA within a CC to be exchanged without restriction, resulting in each lineage sharing many of the same MGEs (e.g. CC1, CC5 and CC8) and has led to the independent evolution of distinct *S. aureus* lineages (Brosnahan et al., 2009, Connelley et al., 2009).

Type II RM systems are endonucleases which target specific palindromic sequences about 4 nt to 8 nt in length. The presence of these enzymes has been reported in *S. aureus* but found to be extremely rare (Bosc and Lefranc, 2000). The Type IV RM systems are the most recent RM systems to have been identified and are widespread in *S. aureus*. They encode a single enzyme gene that can detect the methylation status of a DNA fragment and degrade it (Sriskandan et al., 2001). This system is unlike other restriction modification systems as it will degrade modified DNA rather than unmodified DNA (DaSilva et al., 2002, Yeung et al., 1996). In addition to type I RM, the type IV RM system has presented a major barrier to genetic manipulation of *S. aureus* as DNA introduced directly from *E. coli* is degraded after

transfer (Faulkner et al., 2005, Peavy et al., 1970). Improved understanding of the type IV RM system has allowed the development of *E. coli* strains which amplify plasmids that can be directly transferred from *E. coli* into *S. aureus* without the need for an intermediate host such as *S. aureus* RN4220. This is achieved by directly modifying the DNA so it is not vulnerable to digestion by the type IV modification system (Park et al., 2004, Kelm et al., 1997).

The resistance to foreign elements is also mediated in *S. aureus*, like many other prokaryotes, by clustered regularly interspaced short palindromic repeats (CRISPRs) of which at least 45 loci have been identified (Ballingall et al., 2004). CRISPR sequence with the CRISPR associated protein (Cas) system can identify and degrade exogenous DNA and block phage replication by the production of interfering RNAs (Ellis, 2004, Llewelyn et al., 2006). In *E. coli*, the number and diversity of CRISPR loci had an inverse relationship with the pathogenicity of the isolate tested, which suggests that bacteria with a lower level of CRISPR diversity can acquire more mobile elements but this is balanced against a higher vulnerability to destruction by phage (Elsik et al., 2009). *S. aureus* has also been shown to exploit a mechanism that reduces the lytic activity of bacteriophages and confer resistance to foreign DNA, but still allows the transfer of mobile genetic elements (Le Maréchal C, 2009). The bacteriophage-associated satellite elements, SaPIs can interfere with the transcription of phage genes resulting in the production of a higher number of phage particles containing the SaPI element only, leading to a higher distribution of SaPIs in the bacterial population (Arden et al., 1995).

1.4 Human Immune System

To establish an infection in humans *S. aureus* must interact with the immune system and avoid destruction (Deringer et al., 1997). *S. aureus* has evolved numerous mechanisms

which contribute to avoidance of clearance by the human immune system, these factors will be discussed in detail after first considering some of the key mechanisms deployed in the human host to provide immunity to bacterial pathogens like *S. aureus*. Innate mechanisms such as neutrophil phagocytosis or complement-mediated destruction are the main mechanisms with which the host can clear *S. aureus* before adaptive responses such as T-lymphocytes are employed (Thomas et al., 2009).

1.4.1 Neutrophil granulocyte

The neutrophil or polymorphonuclear leukocyte (PMN) is the most common leukocyte circulating in the mammalian bloodstream and the activity of these cells is the primary mechanism of clearance of *S. aureus* (Seo et al., 2010b). Neutrophils are phagocytes that, when activated by signals such as IL-8, rapidly transmigrate to the site of infection and phagocytose invading organisms (Fig: 1.1) (Arnaud et al., 2004). Neutrophils also contain granules that can be released from the cell or fused with the lysosome (Bateman et al., 2001). They contain a range of enzymes such as elastase or serine proteases which can degrade microbial cells (Wilson et al., 2011). Neutrophils can also release a burst of reactive oxygen species such as hydrogen peroxide which are utilised to kill internalised microbes (Wilson et al., 2011). Another mechanism exploited by neutrophils is the formation of neutrophil extracellular traps (NETs). NETs are formed when the neutrophil releases extracellular DNA which forms a mesh that can trap invading microorganisms (JR Fitzgerald, 1997). When activated, neutrophils release a range of chemoattractants including host defensins that can exhibit antimicrobial functions and can amplify the recruitment of cells such as monocytes (Fitzgerald et al., 2001a). Unlike macrophages, neutrophils undergo apoptosis following phagocytosis which means they do not perform any antigen processing or presentation functions (Kreiswirth et al., 1983). Following apoptosis, neutrophils display an altered cell

surface which signals macrophages to take up the cells along with the degraded microorganisms inside (Holden et al., 2004, Baba et al., 2002). This process is termed efferocytosis and is a key step to clearing an infection and managing the response of neutrophils which can release a wide range of factors into the host that cause damage to host tissues and cells (Kuroda et al., 2001).

1.4.2 Complement

Complement is part of the humoral immune system and is made up of a number of proteins that can directly interact with invading pathogens leading to opsonisation, or killing by the formation of the membrane attack complex (MAC) which creates a destabilising pore in the surface of a pathogen (Kuroda et al., 2001). The Complement system is made up of three different pathways which differ in how they are initially activated; the classical pathway, alternative pathway and the lectin pathway. All three pathways activate homologues of the C3 convertase which cleaves the component C3 into C3a and C3b leading to a cascade of protein cleavage that leaves the surface of the invading cell coated with C3b and C5b opsonins for recognition by phagocytes and clearance (Fig 1.1) (Kuroda et al., 2001). The cleavage of C3 and C5 also release C3a and C5a which are powerful chemoattractants for PMNs (Fig 1.1) (Neoh et al., 2008). Deposition of C5b is also the first step in the formation of the MAC complex which is then followed by the deposition of C6, C7, C8 and then polymeric C9 which forms the majority of the pore (Lowder et al., 2009b). Lysis of the target can occur rapidly, although in nucleated targets the lysis can be resisted, but this leads to cell dysfunction and pro-inflammatory signal release (Diep et al., 2006).

The classical pathway is activated by the binding of either IgM or IgG to the surface of the pathogen, then following this C1q binds to the antibodies and activates the formation of the C1-complex. The C1-complex is made up of C1q and two molecules each of C1r and

C1s. C1q can also bind to the surface of the pathogen independent of the immunoglobulins (Highlander et al., 2007). When formed in the C1complex, C1r, which is a serine protease, cleaves C1s (which is another serine protease) and activates it (Baba et al., 2008). The complex then in turn cleaves C4 and then C2 into C4a, C4b, C2a and C2b leading to the formation of the C4bC2a complex which is the version of C3 convertase used in this pathway (Gillaspy, 2006). Cleaved C3b later joins the C4bC2a to form the C5 convertase (Holden et al., 2004). The lectin pathway is homologous to the classical pathway except instead of antibodies and C1q, activation is initiated by the binding of bacterial carbohydrates by mannose-binding lectins and ficolins (Mwangi et al., 2007).

The alternative pathway is non-specific and does not require the binding of ligands prior to the cascade activation. C3 spontaneously splits by hydrolysis and can bind to hydroxyl or amino groups on the surface of pathogens (Mwangi et al., 2007, Guinane et al.). The cascade can be blocked by host proteins expressed to prevent cascade activation on host cells (Herron-Olson et al., 2007b). If it is not blocked C3b remains on the cell surface and can form the C3bBbp complex by the addition of factor B, D and P and forms the stable form of C3 convertase in this pathway (Gill et al., 2005a). This can cleave more C3 leading to the amplification of the signal on the surface of the pathogen and leads to the activation of the complement cascade (Merle et al., 2015).

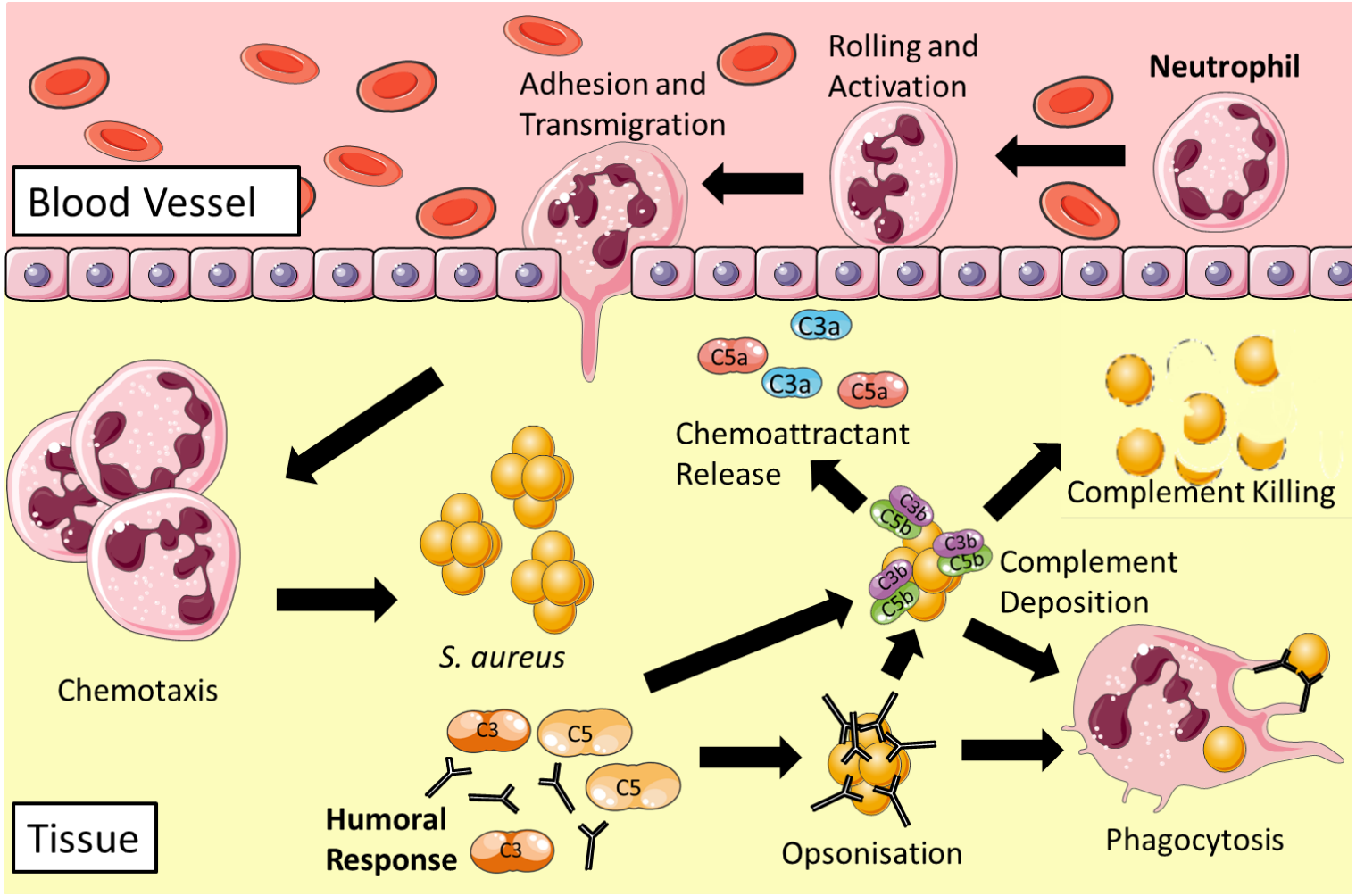
Blood Vessel
Adhesion and Transmigration
Rolling and Activation
Neutrophil
C3a
C5a
C3a
C5a
Chemoattractant Release
Complement Killing
Chemotaxis
S. aureus
C3b
C5b
C3b
C5b
C3b
C5b
Complement Deposition
C3
C5
C5
Humoral Response
C3
Opsonisation
Phagocytosis
Tissue

Figure: 1.1a The role of neutrophils and complement in *S. aureus* clearance. Neutrophils circulating in the blood stream are activated and begin rolling on the surface of the endothelium. Once the cells have adhered to the endothelium they can transmigrate across the barrier and move by chemotaxis towards the site of infection. The humoral response can interact with invading *S. aureus* either opsonising the bacteria for phagocytosis with antibodies or complement products, the bacteria can also be destroyed directly by complement.

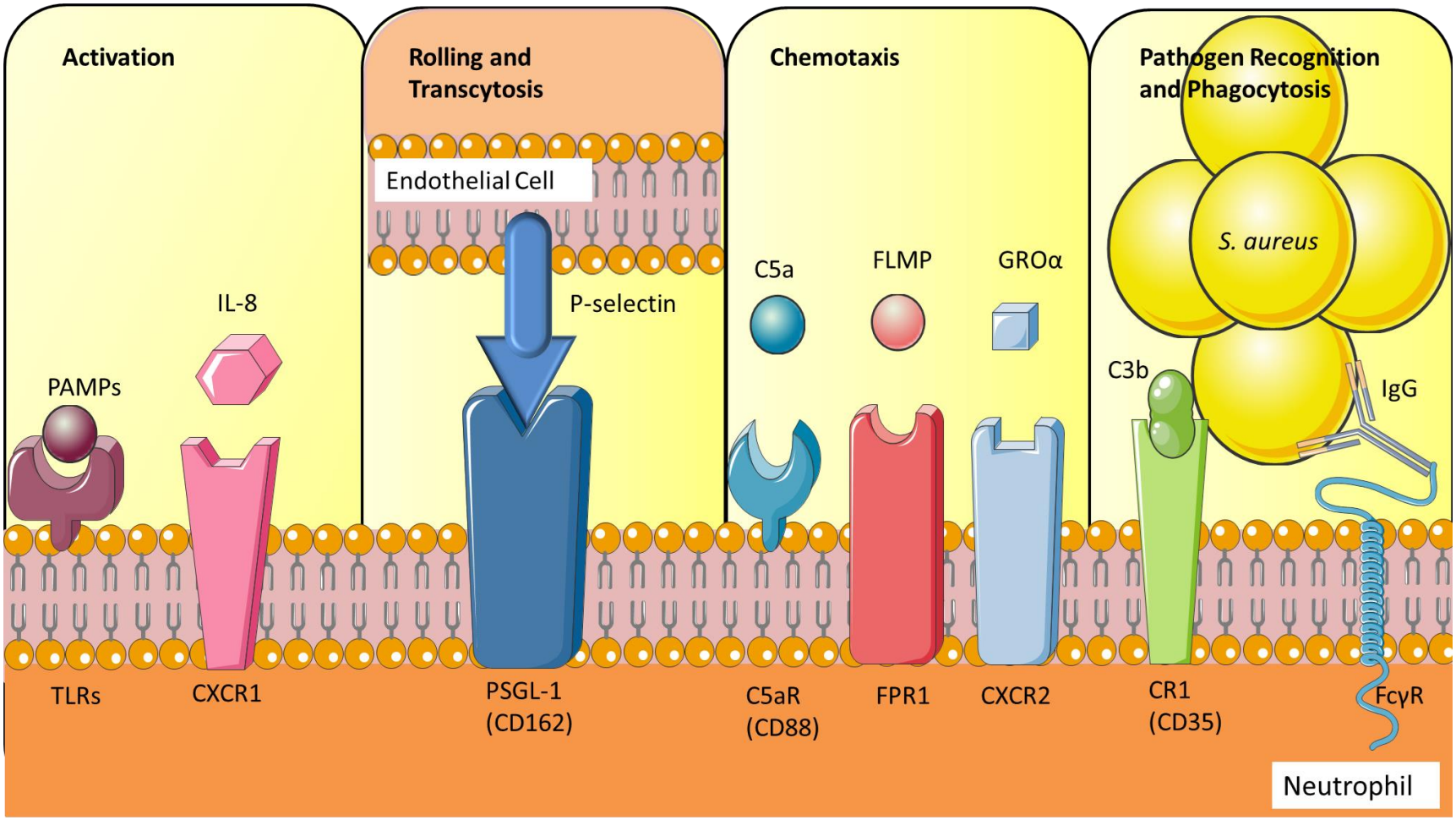
Activation
Rolling and Transcytosis
Chemotaxis
Pathogen Recognition and Phagocytosis
Endothelial Cell
P-selectin
IL-8
PAMPs
C5a
FLMP
GROα
S. aureus
C3b
IgG
TLRs
CXCR1
PSGL-1 (CD162)
C5aR (CD88)
FPR1
CXCR2
CR1 (CD35)
FcγR
Neutrophil

Figure 1.1b The molecular mechanisms involved in neutrophil responses to infection. This figure demonstrates the major molecular interactions involved in the neutrophil response to infection. (**Activation)** A neutrophil can be activated through a range of stimuli including pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) or wall techoic acid (WTA) which are recognised by toll-like receptors (TLRs). Cells can also be activated by cytokines and chemokines such as IL-8 which signals the cell via the chemokine receptor CXCR1. (**Chemotaxis**) IL-8 is also an important chemokine involved in neutrophil chemotaxis along with GROα, pathogen associated molecules such as FLMP (Formyl-Methionyl-Leucyl-Phenylalanine) and anaphlatoxins such as C3a and C5a which are released as a result of complement deposition. (**Rolling and Endocytosis**) A neutrophil must roll along the inside of the endothelium and endocytose through the wall of a blood vessel to reach the site of infection once activated. During the immune response endothelial cells are induced to express P-selectin which binds to PSGL-1 expressed on the surface of neutrophils. This interaction allows the neutrophil to bind to the endothelial wall and begin the transmirgratory process. (**Pathogen Recognition and Phagocytosis**) At the site of infection the neutrophil can detect pathogens opsonised with either antibodies (such as IgG) which are detected by Fc receptors. Alternatively pathogen cell surfaces can be opsonised with deposited complement molecules such as C3b which are recognised by the complement receptor 1 (CR1) receptor. Neutrophils can respond to pathogen recognition by phagocytising the pathogen, releasing neutrophil granules containing antimicrobial compounds, releasing DNA which forms neutrophil extracellular traps (NETs) and releasing cell signalling molecules which recruit other cells in the immune system.

1.4.3 T-lymphocytes

Human T-lymphocytes are an important group of cells which play a critical role in cell mediated immunity (Zhang et al., 2014, Zhu and Paul, 2008). There are many types T-cells which carry out different specialist functions as outlined in Table 1.1. The T-helper or $CD4^+$ T-cells become activated when the T-cell receptor (TCR) binds the major histocompatibility complex (MHC) class II on the surface of an antigen presenting cell (APC) (Krakauer, 2010). Once activated the $CD4^+$ cell can differentiate into a number of subtypes depending on the cytokines present (Fig 1.2). T_h1 T-cell subtypes drive the cellular responses of the immune system by releasing cytokines such as interferon-γ (IFN-γ) and interleukin-2 (IL-2) which maximises macrophage-mediated killing, induces the proliferation of $CD8^+$ cytotoxic T-cells and drives B-cells to produce IgG (Zhang et al., 2014, Zhu and Paul, 2008). If the T-helper cells are stimulated with IL-4 this will drive differentiation into the T_h2 subtype which produces cytokines such as IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 that stimulate cells such as B-cells (to produce IgE), mast cells, basophils and eosinophils producing mediators such as histamine, serotonin and leukotriene (Hammad and Lambrecht, 2015, Zhu and Paul, 2008). Helper T-cells can also interact directly with B-cells via TCR-MHC class II to stimulate the production of antibodies to specific antigens (Crotty, 2011). The cytokines produced by the T_h1 response are inhibitory to the T_h2 response and vice-versa resulting in a bias in the immune response depending on what type of pathogen has activated it i.e. bacteria and viruses are more commonly cleared by the T_h1 response and extracellular pathogens such as helminths trigger the T_h2 response (Hammad and Lambrecht, 2015, Zhang et al., 2014). The T_h17 response is a subset of T-helper cells that differentiate when antigen-activated $CD4^+$ T-cells are exposed to IL-6, IL-21, IL-23 or TGF-β (Bedoya et al., 2013). These cells produce IL-17a and IL-17F along with IL-21 and IL-23 which amplify the T_h17

response (Bedoya et al., 2013). IL-17a and IL-17F are cytokines that have a proinflammatory effect and can induce the productions of other proinflammatory cytokines and chemokines including IL-8 and GRO-α which are chemoattractants of neutrophils (Matsuzaki and Umemura, 2007). It has been demonstrated that IL-17 contributes to *S. aureus* clearance in nasal colonisation by driving the influx of neutrophils (Archer et al., 2013).

Table 1.1 T-lymphocyte types and functions.

T-Cell Type	Molecular Marker	Function	Reference
Helper	CD4	TCR interaction with MHC class II loaded with processed antigen peptides activates specific T-cells and leads to the expression of cytokines that recruits the optimal response depending on which subtype the cells differentiates into.	(Zhang et al., 2014, Zhu and Paul, 2008)
Cytotoxic	CD8	TCR interacts with MHC class I on all nucleated cells. If foreign peptides are detected the T-cell induces apoptotic processes in the infected cell. This is the primary mechanism for removing virally infected or cancerous cells.	(Zhang et al., 2014)
Memory	CD4 or CD8, CD45RO	Subset of antigen specific T-cells that can rapidly activate and expand when antigen specific to the cell is detected. These cells can express either CD4 or CD8 markers and revert to a helper or cytotoxic T-cell when activated. These cells are characterised by a high level of CD45RO expression.	(Zhang et al., 2014, Zhu and Paul, 2008)
Natural Killer T-cells	TCR-invariant chain Vα24-Jα18 and variant Vβ11	These cells recognize foreign/self-lipid presented molecules, such as CD1d, MR1, and CD1a. They act as a bridge between the innate and adaptive immune systems.	(Gao and Williams, 2015)
Suppressor T-cell	CD4	These cells suppress inflammation at the end of an immune response to prevent excessive damage mediated by inflammatory processes.	(Caramalho et al., 2015)
γδ T-cells	TCR γδ	TCR on these cells are made up of a γ and δ chain instead of α and β chains and are not restricted by MHC class I or II interactions. These cells recognize foreign/self-lipid presented molecules. They act as a bridge between the innate and adaptive immune systems.	(Gao and Williams, 2015)

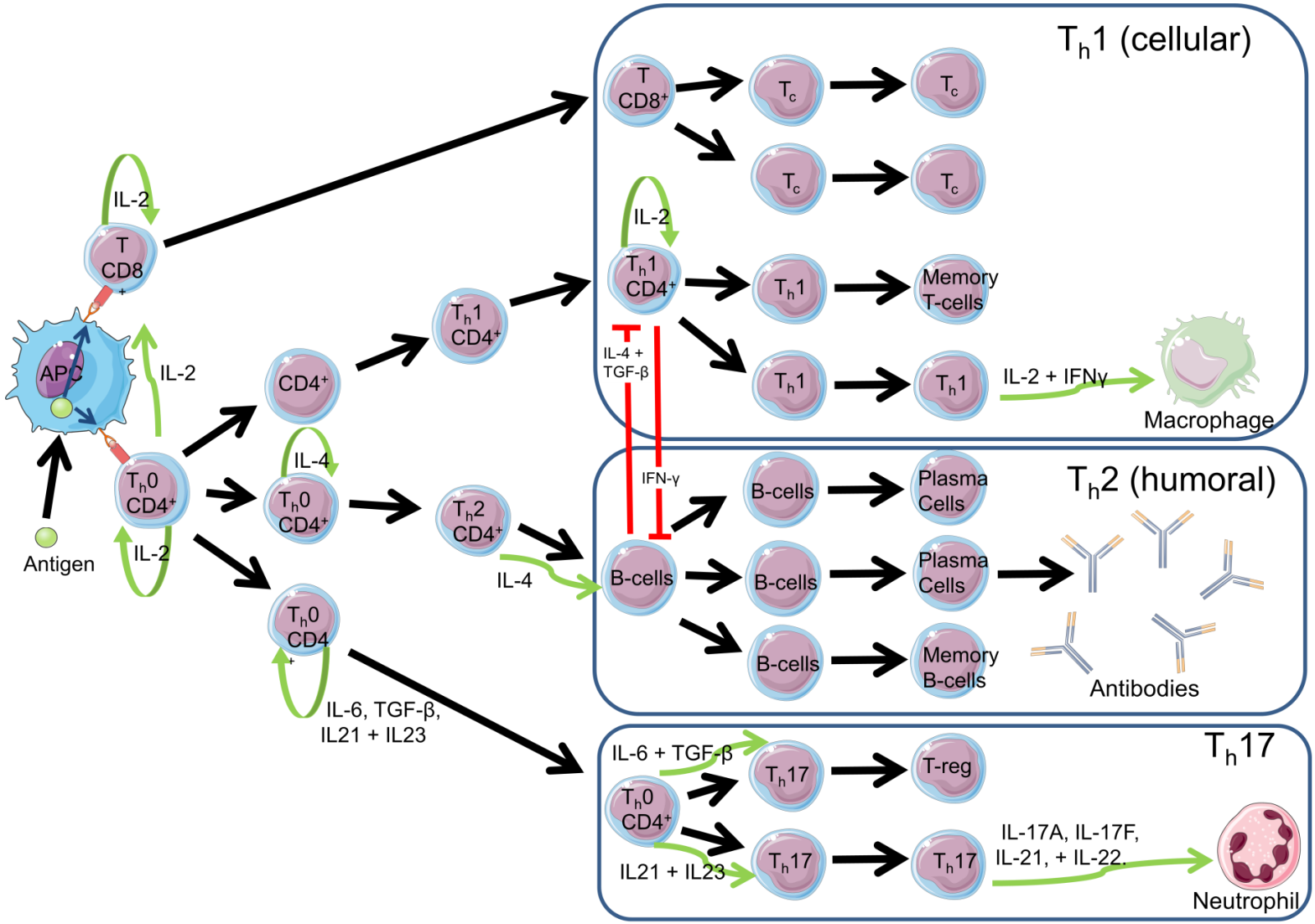
Th1 (cellular)
T CD8+
Tc
Tc
Tc
Tc
IL-2
Th1 CD4+
Th1
Memory T-cells
IL-4 + TGF-β
Th1
Th1
IL-2 + IFNγ
Macrophage
IL-2
APC
IL-2
CD4+
Th1 CD4+
IL-4
Th0 CD4+
Th2 CD4+
IFN-γ
Th2 (humoral)
B-cells
Plasma Cells
Antigen
IL-2
IL-4
B-cells
B-cells
Plasma Cells
B-cells
B-cells
Memory B-cells
Antibodies
Th0 CD4+
IL-6, TGF-β, IL21 + IL23
Th17
IL-6 + TGF-β
T-reg
Th0 CD4+
Th17
IL21 + IL23
Th17
Th17
IL-17A, IL-17F, IL-21, + IL-22
Neutrophil

Figure 1.2 Model of T-cell differentiation after stimulation with antigen. Processed antigen peptide is presented to either $CD4^+$ or $CD8^+$ T-cells by the antigen presenting cell (APC). The stimulation of $CD8^+$ T-cell sub-types with a specific antigen peptide and interleukin 2 (IL-2) results in the differentiation of cells into cytotoxic T-cells (T_c) which kill cells infected with intracellular pathogens. Stimulation of $CD4^+$ T-cells results in the differentiation of T-cells into T_h1 helper T-cells which can produce cytokines such as IL-2 and interferon-γ (IFN-γ) which helps coordinate the T_h1 cellular response. Exposure to IL-4 during $CD4^+$ T-cell stimulation drives differentiation of T-cells into T_h2 helper cells which help activate B-cells to produce IgE. T_h2 responses can be repressed by the IFN-γ when T_h1 response is activated and the T_h1 response is repressed by the release of IL-4 and transforming growth factor-β when the T_h2 response is activated. The T_h17 response is activated by the exposure of $CD4^+$ T-cell exposure to IL-6, IL-21, IL-23 or TGF-β. Stimulation of the T_h17 cells with IL-6 and TGF-β drives the differentiation of regulatory cells and the factors IL-21 and IL-23 drive the differentiation of proinflammatory T_h17 which includes the production of IL-17A and IL-17-F which recruits and activates components of the innate immune response including neutrophils.

1.5 *S. aureus* pathogenesis

S. aureus expresses a diverse array of both secreted and surface expressed virulence determinants. These factors allow *S. aureus* to adhere to the extracellular matrix molecules, spread in the host and evade the host immune response.

1.5.1 Enzymes

S. aureus secretes a wide range of exotoxins which enable the bacterium to survive in the host and contribute to virulence. These exotoxins include a large number of degradative enzymes such as nucleases, proteases and lipases which can be utilised by *S. aureus* to facilitate spread in the host by degrading tissue constituents (Koziel and Potempa, 2013). Examples of this are the cysteine proteases staphopain A and B which degrade fibrinogen and collagen, impair plasma clotting and may play a role in damaging the endocardium, the initial stage in the progression to endocarditis (Ohbayashi et al., 2011) Another protease that has been shown to play an important role in *S. aureus* pathogenesis is the metalloprotease, aureolysin (Cassat et al., 2013). Aureolysin, along with staphopain A and B and the serine protease V8 have been shown to interact with components of the complement system and contribute to the avoidance of complement killing (Jusko et al., 2014, Laarman et al., 2011). The protease-complement interaction may also contribute to *S. aureus* colonisation as V8 and aureolysin interaction with C1q promotes deposition of C1q on the surface of *S. epidermidis* but not *S. aureus* giving *S. aureus* an advantage against this competitor (Jusko et al., 2014).

Nucleases can be used by *S. aureus* to degrade DNA in neutrophil extracellular traps (NETs), which are deployed by neutrophils to restrict bacterial spread (Berends et al., 2010, Thammavongsa et al., 2013). *S. aureus* can also use the degradation of NETs to induce macrophage cell death, NETs are cleaved by nuclease and adenosine synthase which

produces deoxyadenosine and exposure to this molecule triggers the caspase-3 pathway in macrophages leading to macrophage apoptosis (Thammavongsa et al., 2013).

Staphylocoagulase and vWBP can interact with the host enzyme, prothrombin, and manipulate its activity (McAdow et al., 2012). Staphylocoagulase and vWBP can both bind to prothrombin and force a conformational change in prothrombin generating the formation of the staphylothrombin complex (Friedrich et al., 2003, Kroh et al., 2009). Staphylothrombin converts fibrinogen to fibrin which generates the formation of infected thrombi and assists in the establishment of infection in the host, and binding to the wall of blood vessels in a systemic infection (Claes et al., 2014, Panizzi et al., 2006, Bjerketorp et al., 2004). The fibrinogen conversion can also activate platelets which bind to fibrin formed by staphylothrombin and results in platelet aggregation (Vanassche et al., 2012). *S. aureus* can then interact directly with the platelets and establish infected thrombi (Vanassche et al., 2012). The establishment of an infected fibrin network can modify the host response protecting *S. aureus* from direct interactions with leukocytes and allowing the bacteria to aggregate in a focal point which allows *S. aureus* to persist *in vivo* longer in comparison to a coagulase-deficient isogenic mutant (Loof et al., 2015, Vanassche et al., 2011).

1.5.2 α-Haemolysin

S. aureus also secretes other important exotoxins including the cytolytic toxins α-, β, γ- and δ-toxins which can lyse neutrophils aiding *S. aureus* evasion of the immune response (Alonzo and Torres, 2014). The α-toxin (Hla) secreted by *S. aureus* is a pore-forming toxin which is secreted as a single monomer and these monomers then assemble into a heptamer in the membrane of the target cell forming a β-barrel pore in the cell membrane resulting in cellular leakage and eventually lysis (Berube and Bubeck Wardenburg, 2013). This toxin has a wide target range of mammalian cells including monocytes and endothelial cells (Bhakdi

and Tranum-Jensen, 1991). It is critical to *S. aureus* pathogenesis and is encoded by the great majority of clinical isolates of *S. aureus* (Berube and Bubeck Wardenburg, 2013). The protein target for Hla is the ADAM-10 (a disintegrin and metalloprotease 10), a zinc-dependent metalloprotease expressed as a transmembrane protein on a number of cell types, which explains how Hla can target a large number of cell types (Wilke and Bubeck Wardenburg, 2010). ADAM-10 binding by Hla allows the formation of the toxin pore which has been shown to be critical for Hla-induced tissue injury (Wilke and Bubeck Wardenburg, 2010, Inoshima et al., 2011).

Tissue disruption and damage to barriers such as skin, lung mucosa and vasculature is a feature of staphylococcal disease and much of this damage is attributed to the activity of Hla (Berube and Bubeck Wardenburg, 2013). The pore formed by the toxin can interfere with signalling pathways by causing an influx of Ca^{2+} ions into cells (Inoshima et al., 2011). The interaction of Hla with ADAM10 can also cause modifications to E-cadherin which together with pore formation can alter tight junctions and alter the integrity of epithelial and endothelial barriers (Powers et al., 2012). Hla interaction with ADAM10 can also affect endothelial barriers by inducing inflammation via neutrophil activation and altering platelet activation. Inflammation of the endothelium can result in the reduction of wound repair and the formation of PMN-platelet aggregations in organs such as the lung leading to lethal sepsis (Powers et al., 2015). Hla has also been shown to contribute to the escape from the macrophage phagosome and then can prevent apoptosis of the infected cells by inducing the production of anti-apoptotic mediators such as myeloid cell leukemia-1 (MCL-1) allowing *S. aureus* to survive and disseminate in an infected macrophage (Koziel et al., 2015).

1.5.3 Leukocidins

Leukocidins are toxins secreted by *S. aureus* as two distinct monomers, a slow (S) subunit and a fast (F) subunit based on the original purification of PVL (Finck-Barbancon et al., 1991). When the monomers bind to their targets on the cell surface they assemble into an octomeric pore (composed of 4 F-subunits and 4 S-subunits) (Yoong and Torres, 2013). γ-toxin or HlgACB (expressed as either HlgAB or HlgBC) is a bi-component leukotoxin which is secreted as HlgA/C (S-subunit) and HlgB (F-subunit), the HlgA/C subunit binds to the cell first and then this is followed by the binding of the HlgB subunit (Kaneko et al., 1997). HlgACB is the only leukocidin that can lyse erythrocytes efficiently and is highly upregulated when *S. aureus* is incubated in blood, promoting survival (Malachowa et al., 2011). HlgACB is encoded in most clinical isolates suggesting a critical role in pathogenesis (von Eiff et al., 2004) and has shown to be important in a murine model of sceptic arthritis demonstrating a role *in vivo* (Nilsson et al., 1999).

Other bi-component toxins include the leukocidin A/B and E/D (LukAB and LukED). LukAB was recently described and has been demonstrated to lyse neutrophils, monocytes, macrophage and dendritic cells (Dumont et al., 2011, Ventura et al., 2010, DuMont et al., 2013, Malachowa et al., 2012). LukAB is a bi-component cytotoxin which targets neutrophils by binding to the CD11b component of the MAC-1 complex (DuMont et al., 2013). Unlike other *S. aureus* bicomponent leukocidins LukAB forms heterodimers in solution rather than remaining as a monomer and the S subunit (LukA) includes extensions at the N- and C-terminus by 33 and 10 amino acids respectively (DuMont et al., 2014, Dumont et al., 2011, Badarau et al., 2015). Truncation of the C-terminal domain of LukA rendered the toxin inactive and more detailed study revealed a glutamic acid residue at position 323 was critical to toxicity, whereas deletion of the N-terminal domain only had a modest impact on cytotoxicity (DuMont et al., 2014). LukAB is the only leukocidin so far that has been reported to lyse neutrophils and monocytes after phagocytosis suggesting that

this toxin may contribute to escape from the phagosome (Dumont et al., 2011, Melehani et al., 2015).

LukED targets neutrophils, lymphocytes, macrophages and dendritic cells by binding the CCR5, CXCR1 or CXCR2 receptors and by targeting these cells can promote *S. aureus* survival during infection (Reyes-Robles et al., 2013, Alonzo et al., 2013). This toxin has been shown to promote bacterial growth in a murine model by targeting and destroying murine neutrophils, whereas deletion of the *luk*ED genes or depletion of murine neutrophils abrogated the effect (Alonzo et al., 2012). LukED is highly conserved (100% amino acid identity found in all strains encoding this gene) and found in ~87% of clinical strains suggesting an important role in *S. aureus* pathogenesis (Alonzo et al., 2012). Another Leukocidin (LukMF') was reported recently with specific toxicity for bovine neutrophils. The species-specificity is due to the binding partner of LukMF', CCR1, which is expressed at high levels on bovine neutrophils but not on human neutrophils (Vrieling et al., 2015).

PVL is another example of a bi-component β-pore forming cytotoxin which acts primarily on neutrophils (Kaneko and Kamio, 2004, Yoong and Torres, 2013). PVL is present in almost all CA-MRSA clones and heavily associated with necrotising skin lesions and pneumonia in CA-MRSA infections (Gillet et al., 2002, Bocchini et al., 2006, De Boeck et al., 2015). However some controversy surrounded the role of this toxin as contradictory evidence regarding its role in pathogenesis had been obtained from different animal models. In particular, murine models of infection had produced limited observable effects for this toxin (Diep and Otto, 2008, Voyich et al., 2006). Importantly PVL has been shown to be species-specific inducing rapid activation and cell death in human and rabbit neutrophils but not murine or simian cells (Löffler et al., 2010). Studies in rabbits have shown that PVL expression leads to more severe skin lesions (Lipinska et al., 2011), more severe and persistent osteomyelitis (Cremieux et al., 2009) and induces lung necrosis by activating and lysing PMNs (Diep et al., 2010). The target for PVL was identified as the C5a receptor on

the surface of neutrophils (Spaan et al., 2013). C5a exhibits variation among the animal species employed to investigate the role of PVL and explains the variation seen in these different studies (Spaan et al., 2013, Spaan et al., 2015). The host-specificity of PVL highlights the importance of understanding the molecular mechanisms involved in pathogenesis and using this information to select the most appropriate animal model. PVL can act synergistically with other haemolysins including β-toxin, LukED, γ-toxin and δ-toxin and amplify both the cytolytic and proinflammatory effect of these toxins (Perret et al., 2012). PVL can also induce the apoptosis of PMNs by targeting the mitochondria of these cells, PVL was shown to localise to the mitochondria in the cytosol in PMNs and it is suggested that the toxin forms pores in the mitochondria resulting the release of apoptogenic proteins cytochrome c and Smac/DIABLO (Genestier et al., 2005).

Studies on the sublytic concentrations of leukocidins have indicated that these toxins have pro-inflammatory properties (Alonzo and Torres, 2014). Sublytic concentrations of the secreted toxins Hla, HlgACB, PVL and LukAB have all been found to activate the intracellular NOD-like Receptor (NLR) protein 3 (NLRP3) inflammasome in neutrophils, monocytes and macrophages leading to activation of Caspase 1 pro-inflammatory cytokines such IL-1β and IL-18, and induction of necrotic cell death (Davis et al., 2011, Holzinger et al., 2012, Melehani et al., 2015, Kebaier et al., 2012, Munoz-Planillo et al., 2009). This demonstrates that inflammation can be trigged by these toxins independent of pore formation (Holzinger et al., 2012). In a murine model, Hla induction of this caspase-1 inflammatory pathway was shown to induce abscess formation and lead to clearance of *S. aureus* (Cho et al., 2012). Conversely in the murine pneumonia model, the caspase-1 pathway activated by Hla resulted in more severe pathology (Kebaier et al., 2012). The activation of the NLRP3 inflammasome is clearly an important function of haemolysins and leukocidins as these toxins overlap in their function (Yoong and Torres, 2013). However despite the functional overlap, it is not clear how the induction of inflammation enhances bacterial survival, one

possibility is that under certain conditions, inflammation and the associated tissue damage may allow *S. aureus* spread to different sites within the host (Vandenesch et al., 2012).

1.5.4 Phenol soluble modulins

Phenol soluble modulins (PSMs) are a group of short peptides made up of two groups, α-PSMs (20-25 amino acids) and the longer β-PSMs (~44 amino acids) (Otto, 2014). In *S. aureus* 7 PSM peptides have been identified including δ-toxin (Otto, 2014). These short peptides form α-helices and this degree of helix formation appears to relate to the lytic potential of the peptide (Laabei et al., 2014). Increased production of PSMs has been associated with clinical isolates of MRSA from skin and soft tissue infections (SSTIs) and suggests these peptides play an important role in the pathogenesis of SSTIs (Berlon et al., 2015). The lytic activities of these peptides are readily inactivated in serum which suggests that extracellular cell lysis does not have a role during infection (Surewaard et al., 2012). These peptides have been shown to be expressed inside the phagosome, in PMNs, facilitating *S. aureus* escape from the phagosome and lysing cells from the inside (Surewaard et al., 2013, Grosz et al., 2014). PSM peptides have been shown to induce the release of pro-inflammatory cytokines from a range of cells including dendritic cells (DC) and regulatory T-cells (T-regs). Stimulation of DCs and T-regs in this way can lead to the release of the IL-10 resulting in the dampening of the immune response (Schreiner et al., 2013). The PSM δ-toxin was also shown to activate mast cells by inducing calcium flux into the cell which results in the release of mast cell granules promoting IgE and IL-4 production (Nakamura et al., 2013). Allergic skin diseases are characterised with a high level of δ-toxin and the dysregulation of mast cells which may provide a mechanism for the link between *S. aureus* colonisation and allergic skin disease (Nakamura et al., 2013)

PSMs have also been suggested to play a role in prosthetic joint infections. In the early stages of biofilm formation the expression of PSMs is low due to the low activity of the *agr* locus (Queck et al., 2008, Dastgheyb et al., 2015). As cell numbers increase PSMs are expressed and act as surfactants disrupting interactions between polysaccharide molecules such as polysaccharide intercellular adhesin (PIA) resulting in release of the bacteria from the biofilm and dissemination in the host (Dastgheyb et al., 2015). PSMs are also thought to play a key role in colonisation owing to their surfactant properties aiding growth at an oil/water interface (Otto, 2014). PSMs have shown to aid the spread of *S. aureus* growth over a wet surface aiding colonisation (Tsompanidou et al., 2013). Furthermore, PSMs have been demonstrated to contribute to the formation of extracellular fibrils which stabilise *S. aureus* biofilms (Schwartz et al., 2012). There is also some evidence to suggest that PSMs may have antimicrobial activity as PSMα1 and PSMα2 have been demonstrated to exhibit anti-streptococcal activity, this may assist *S. aureus* to out-compete other bacteria at the site of colonisation (Joo et al., 2011).

1.5.5 Other secreted immune modulatory proteins

S. aureus secretes a battery of immunomodulatory proteins that can interfere with the function of both complement and neutrophils. Chemotaxis inhibitory protein of staphylococcus (CHIPS) functions by binding the formylated peptide receptor and the C5a receptor on neutrophils, blocking cheomtaxis to the site of infection (Postma et al., 2004). SCIN interacts with C3 convertases bound to the bacterial surface and prevents C3b deposition and phagocytosis (Rooijakkers et al., 2005). CHIPS and SCIN are both expressed at an early stage during *S. aureus* infection and blocks essential steps in the acute immune response (Rooijakkers et al., 2006). The formyl peptide receptor-like 1 inhibitor (FLIPr) and its homologue FLIPr-like have been shown to both have anti-inflammatory properties by

antagonising the formylated peptide receptor but also can bind to Fcγ receptors and interfere with IgG-mediated phagocytosis of neutrophils (Prat et al., 2006, Stemerding et al., 2013). The extracellular adherence protein (EAP) can mediate interaction between the bacterial cell wall and matrix proteins such as fibronectin and has also been shown to have a number of immunomodulatory effects such as the inhibition of the activity of neutrophil serine proteases including neutrophil elastase (Stapels et al., 2014, Geisbrecht et al., 2005). EAP has also been shown inhibit both the lectin and classical complement pathways by binding to the C4b component and blocking the formation of the C3 convertase (Woehl et al., 2014). EAP has also been shown inhibit both the lectin and classical complement pathways by binding to the C4b component and blocking the formation of the C3 convertase (Woehl et al., 2014). The extracellular fibrinogen binding protein (Efb) of *S. aureus* can bind to fibrinogen and prevent platelet aggregation (Shannon and Flock, 2004). Efb can also bind to complement component C3 simultaneously with plasminogen which in turn leads to the degradation of C3, this functional process is also seen in surface immunoglobulin-binding protein (Sbi) and contributes to the inactivation and removal of C3b from the surface of the bacteria (Koch et al., 2012). The combination of complement and fibrinogen binding interactions for Efb is suggested to create an 'immune capsule' around the bacteria which can reduce opsonophagocytosis of *S. aureus* (Ko et al., 2013).

1.5.6 Staphylococcal superantigen-like proteins

One of the largest groups of immune modulators is the family of 14 staphylococcal superantigen-like toxins (SSLs) which share a similar structure to staphylococcal superantigens (SAgs), but lack mitogenic activity (Fraser and Proft, 2008b). Instead they appear to have alternative roles in modulating the immune response (Table 1.2) (Thammavongsa et al., 2015). The *ssl* genes 1-11 are found in the VSaα region which is a

highly variable region of the chromosome of *S. aureus*. This region has been identified in all strains so far and can vary in size from 12 to 17 kB encoding at least 7 of the *ssl* genes (Fitzgerald et al., 2001b, Fraser and Proft, 2008b, Kuroda et al., 2001). The other three *ssl* genes are found at a distinct genomic region 0.7 MB upstream of vSaα (Fraser and Proft, 2008b, Kuroda et al., 2001).

The SSl-proteins studied so far have shown a range of functional properties that include inhibition of both the humoral and cellular immune response. For example, SSl7 was demonstrated to bind to complement factor C5 and the immunoglobulin IgA, and is therefore suspected to play a role in immune evasion in the mucosa (Langley et al., 2005, Bestebroer et al., 2010). SSl7 was shown to enhance the survival in blood of *Lactococcus lactis* when SSl7 was transformed into the bacteria (Lorenz et al., 2013). SSl7 was shown to reduce neutrophil migration in a peritoneum infection model with heat-killed *S. aureus* and C5 binding capacity of SSl7 was shown to be critical to this function as a C5-binding mutant was incapable of blocking the migration of neutrophils (Lorenz et al., 2013). SSl10 can interact directly with IgG and block the association of C1q, a critical component in the classical complement cascade (Itoh et al., 2010b), whereas SSl8 binds to tenascin-C and attenuates the interaction between this molecule and fibronectin (Itoh et al., 2013). If tenascin-C cannot interact with fibronectin, keratinocyte motility is reduced and this effect reduces the speed of wound recovery (Itoh et al., 2013).

To date, SSl5 is the best characterised neutrophil-binding SSl-protein with a number of distinct functions identified. For example, SSl5 can bind to neutrophils via the P-selectin glycoprotein ligand-1 (PSGL-1) which is used by the neutrophil to facilitate rolling on the surface of the endothelium prior to transmigration outside of the blood vessel (Bestebroer et al., 2007). The interaction of SSl5 with this target blocks neutrophil rolling, inhibits platelet adhesion and can reduce the formation of thrombi (Armstrong et al., 2012, Walenkamp et al., 2010). The binding of SSl5 can also have an effect on neutrophil activation by inhibiting

calcium mobilisation after stimulation with the chemokines C3a, C5a and CXCL8 (Bestebroer et al., 2009). SSl5 can also bind factors secreted by activated neutrophils such as the matrix metalloprotease 9 (MM9) which SSl5 can bind to and inhibit (Itoh et al., 2010a). Inhibition of MM9 may affect neutrophil migration as this enzyme is involved in regulation and migration of neutrophils over the basement membrane (Opdenakker et al., 2001). SSl5 contains a binding motif that shares homology with conserved sialic acid binding proteins from other bacteria and viruses and can interact with human glycans (Baker et al., 2007). This binding site was found to be conserved in SSl2, SSl3, SSl4 SSl6 and SSl11 suggesting that these proteins can bind glycans expressed on immune cells (Baker et al., 2007). Despite these shared glycan binding sites being similar between these SSl-proteins, they do exhibit differential binding to glycans which suggests they have distinct tropisms to receptors expressed on the surface of cells (Baker et al., 2007, Hermans et al., 2012, Chung et al., 2007).

Functions identified for other neutrophil binding SSl-proteins include TLR-2 antagonism by SSL3, and induction of phagocytosis of erythrocytes by SSl6 binding via CD47 (Bardoel et al., 2012, Fevre et al., 2014, Koymans et al., 2015). One feature of SSl-proteins 4, 5 and 11 is that following glycan binding they are rapidly internalised into vesicles in neutrophils and macrophages (Chung et al., 2007, Hermans et al., 2012), which may lead to functional interference within the phagocyte.

Table 1.2 Staphylococcal superantigen-like (SSl) proteins, ligands and proposed functions.

SSl-protein	Binding Target(s)[a]	Proposed Function[a]	Reference
1	NK	NK	
2	NK (Predicted glycan binding motif)	NK	(Baker et al., 2007)
3	TLR2[b]	TLR2 signalling inhibition	(Bardoel et al., 2012, Koymans et al., 2015, Yokoyama et al., 2012)
4	sLEX[c] (CD15)	NK	(Hermans et al., 2012)
5	PSGL-1, GPCRs, GPIbα, MM9 and GPVI	Neutrophil chemotaxis and platelet inhibition	(Armstrong et al., 2012, Baker et al., 2007, Bestebroer et al., 2007, De Haas et al., 2009, Itoh et al., 2010a)
6	CD47	Chemotaxis inhibition	(Fevre et al., 2014)
7	IgA, C5	Phagocytosis inhibition, complement inhibition	(Bestebroer et al., 2010, Langley et al., 2005)
8	Tenascin A	Decreased wound healing	(Itoh et al., 2013)
9	NK	NK	
10	IgG, fibrinogen, fibronectin, thrombin and factor Xa	Phagocytosis inhibition	(Itoh et al., 2010b, Patel et al., 2010)
11	sLEX (CD15), FcαRI and PSGL-1	Chemotaxis inhibition	(Chung et al., 2007)
12	NK	NK	
13	NK	NK	
14	NK	NK	

[a] NK – not known [b] TLR2 – Toll like receptor 2 [c] sLEX – sialic lewis X

1.5.7 Cell wall anchored proteins

S. aureus encodes a diverse range of cell wall anchored (CWA) proteins that contribute to pathogenesis and to date at least 24 different proteins have been identified (Foster et al., 2014). These proteins are important virulence factors as they can be used to adhere to host tissue components, bind proteins in blood, mediate host cell invasion and contribute to resistance against phagocytosis (Foster et al., 2014). Many CWA proteins bind extracellular matrix molecules and as such are termed MSCRAMMs (matrix surface components recognising adhesive matrix molecules) (Foster and Hook, 1998). The major MSCRAMMs that have been characterised (Table 1.3) include clumping factors (Clf) A and B and fibronectin binding proteins (FnBP) A and B. Other groups of CWA proteins include the near iron transporter (NEAT) motif family, three helical bundle family and the G5-E repeat family (Table 1.3) (Foster et al., 2014).

FnBPA and B both bind fibronectin, fibrinogen and elastin which are recognised and bound by the GPIIb/IIa integrin on resting platelets and can result in platelet activation and aggregation (Fitzgerald et al., 2006, Keane et al., 2007). *S. aureus* internalisation has also been shown to be dependent of FnBPs (Sinha et al., 2000). The FnBPs have also been associated with the formation of *S. aureus* biofilms and it is proposed that bacterial cell to cell interactions mediated via the FnBPs result in the accumulation of cells leading to the formation of a biofilm (McCourt et al., 2014, Herman-Bausier et al., 2015)

Staphylococcal protein A (SpA) is a CWA protein that has been classically shown to bind the Fc region of IgG blocking the interaction between IgG and the Fcγ receptor on phagocytic cells (Deisenhofer, 1981). Blocking the Fc region prevents the opsonisation of the bacterial cell and reduces the rate of phagocytosis (Clarke and Foster, 2006). SpA has also been shown to bind von Willebrand factor (vWF) (a serum protein that mediates platelet adhesion at sites of endothelial damage) which may contribute to the formation of infected

thrombi (Hartleib et al., 2000, O'Seaghdha et al., 2006). SpA can also be released from the cell wall into the extracellular matrix allowing the protein to interact directly with human B-cells (Becker et al., 2014). SpA can bind to the human V_H clan III-encoded (V_H3) B-cell receptor (BCR) which makes up about 50 % of V_H genes and allows SpA to interact with over 30% of human B-lymphocytes (Silverman et al., 1993, Graille et al., 2000). This interaction of SpA and the BCR appears to drive the proliferation of V_H3 B-lymphocytes (Kristiansen et al., 1994). This has been shown to occur *in vitro,* and supraclonal expansion of V_H3 B-cell can also been observed in the murine sepsis model (Palmqvist et al., 2005). The superantigenic function of SpA can induce clonal deletion of non V_H3 B-cell subsets and immunodominance of the V_H3 subset which limits the host response to *S. aureus* infection by diminishing the B-cell repertoire (Pauli et al., 2014). SpA is critical to *S. aureus* pathogenesis as the IgG binding and B-lymphocytes interactions help *S. aureus* avoid immune surveillance and prevent the maturation of B-cell subsets that produce immunoglobulins that can inactivate or opsonise *S. aureus* (Falugi et al., 2013).

ClfA is a protein that can bind fibrinogen and is the dominant protein involved in activation of platelets at the stationary phase of growth, contributing to the formation of infected thrombi (Foster et al., 2014). These molecules can engage resting platelet glycoprotein GPIIb/IIIa, aided by bound IgG molecules triggering activation of GPIIb/IIIa and aggregation of platelets (Loughman et al., 2005). A ClfA mutant, unable to bind fibrinogen, demonstrated that ClfA is able to induce platelet activation independent of fibrinogen by the deposition of complement (Loughman et al., 2005). Complement association of ClfA can also contribute to immune evasion as ClfA can bind to complement control factor 1 and increase C3b cleavage on the surface of the *S. aureus* cell wall resulting in reduced opsonophagocytosis and complement-mediated killing (Hair et al., 2010). Like ClfA, ClfB also binds fibrinogen and can meditate the activation of resting platelets (Miajlovic et al., 2007). Unlike ClfA, ClfB is only expressed in early exponential growth

phase, can bind cytokeratin 10 and plays a role in host colonisation on the skin and in the nasal passage (Weidenmaier et al., 2012, Schaffer et al., 2006, Mulcahy et al., 2012). It has also been shown that ClfB can contribute to biofilm formation in the absence of calcium (Abraham and Jefferson, 2012). By binding host fibrinogen, ClfA and ClfB may aid in the evasion of the host immune system by restricting the access of opsonins to the bacterial cell surface (Foster, 2005, Higgins et al., 2006).

In addition to ClfA a number of CWA proteins have been shown to bind to components of the complement system. The collagen binding protein (Cna) interacts with both collagen in the extracellular matrix and has also been shown to be able to interact with C1q from the classical pathway of the complement system reducing complement activation in response to *S. aureus* infection (Kang et al., 2013). Bone sialoprotein-binding protein (BbP) can bind to the C4b binding protein and can inhibit bacterial killing and opsonisation by reducing the association of C3 complement fragments on the surface of the bacterial cell wall (Hair et al., 2013). The iron uptake protein IsdH has also been associated with complement modification, although no ligand has been identified (Visai et al., 2009). A deletion mutant of IsdH in *S. aureus* demonstrated reduced virulence in a sepsis model and was engulfed at a higher rate by human neutrophils, these observations were linked to a higher level of C3b degradation on the surface of *S. aureus* in the presence of IsdH (Visai et al., 2009). Serine-aspartate repeat protein E (SdrE) can also manipulate complement activation by binding to complement factor H which resulted in a higher level of conversion of C3b to iC3b (inactive C3b) on the surface of the bacterial cell and preventing the activation of the complement cascade (Sharp et al., 2012).

Table 1.3 Major characterised CWA proteins from *S. aureus* and their ligands (adapted from Foster et al. (2014)).

CWA protein	Ligands	Function	Reference
MSCRAMMs			
Bone sialoprotein-binding protein (Bbp)	Bone sialoprotein, C4b binding protein, fibrinogen	Adhesion to ECM, Immune evasion by blocking C3 deposition	(Hair et al., 2013, Vazquez et al., 2011)
Clumping Factor A (ClfA)	Fibrinogen and complement regulator factor I	Adhesion to ECM, Immune evasion by degradation of C3b	(Hair et al., 2010, Ganesh et al., 2008)
Clumping Factor B (ClfB)	Fibrinogen, loricrin and cytokeratin 10	Adhesion to ECM, Nasal colonisation	(Ganesh et al., 2011, Xiang et al., 2012)
Collagen Binding Protein (Cna)	Collagen and C1q	Adhesion to collagen rich tissue, immune evasion by prevention of classical complement pathway activation	(Ross et al., 2012, Kang et al., 2013)
Fibronectin Binding protein A (FnBPA)	Fibronectin, fibrinogen and elastin	Adhesion to ECM, host cell invasion	(Keane et al., 2007, Stemberk et al., 2014, Bingham et al., 2008)
Fibronectin Binding Protein B (FnBPB)	Fibronectin , fibrinogen and elastin	Adhesion to ECM, host cell invasion	(Roche et al., 2004, Burke et al., 2010)
Serine-aspartate repeat protein C (SdrC)	Desquamated epithelial cells and β-neurexin	Potential role in nasal colonisation	(Corrigan et al., 2009, Barbu et al., 2010)
Serine-aspartate repeat protein D (SdrD)	Desquamated epithelial cells	Potential role in nasal colonisation	(Corrigan et al., 2009)
Serine-aspartate repeat protein E (SdrE)	Complement Factor H	Immune evasion by degradation of C3b	(Sharp et al., 2012)

CWA protein	Ligands	Function	Reference
Three Helical Bundle			
Staphylococcal Protein A (SpA)	IgG, IgM (B-cell receptor) and von Willebrand factor	Opsonophagocytosis avoidance, B-cell superantigen and a mediator of endovascular infection	(O'Seaghdha et al., 2006, Foster, 2005)
NEAT motif family			
Iron Regulated Surface determinant A (IsdA)	Haem, cytokeratin 10, fibronectin and fibrinogen	Iron uptake, adhesion to desquamated epithelial cells, resistance to lactoferrin immune evasion by conferring resistance to host defensins and antibacterial lipids	(Clarke et al., 2004, Clarke et al., 2007, Clarke et al., 2009, Clarke and Foster, 2008)
Iron Regulated Surface determinant B (IsdB)	Haem, Haemoglobin and β3 integrins	Iron Uptake and invasion of host cells	(Miajlovic et al., 2010, Pishchany et al., 2014, Zapotoczna et al., 2013, Pilpa et al., 2009)
Iron Regulated Surface determinant H (IsdH)	Haem, haemoglobin and an unknown complement component	Iron uptake and accelerated degradation of C3b	(Visai et al., 2009, Pilpa et al., 2009)
G5-E repeat family			
S. aureus surface protein G (SasG)	Not determined	Adhesion to desquamated epithelial cells and biofilm formation	(Roche et al., 2003, Geoghegan et al., 2010)
Plasmin-sensitive surface protein (Pls)	Not determined	Adhesion to desquamated epithelial cells	(Roche et al., 2003)

1.5.8 *S. aureus* host cell invasion

S. aureus has conventionally been considered to be an extracellular pathogen, but in 1986 it was first observed internalised in non-professional phagocytes (Hamill et al., 1986, Vann et al., 1986). *S. aureus* internalisation has been found to be mediated by FnBPA and B by forming a bridge via fibronectin to host cell α_5-β_1 integrin (Foster et al., 2014). Extracellular adherence protein (EAP) has also been demonstrated to be an invasin and can promote the invasion of *S. aureus* and other staphylococci into keratinocytes (Sinha and Fraunholz, 2010, Bur et al., 2013). The iron regulated surface determinant B (IsdB) can also mediate *S. aureus* invasion into host cells independent of fibronectin and the fibronectin binding proteins of *S. aureus* (Zapotoczna et al., 2013).

Once *S. aureus* has been internalised into the host cell it can survive for a prolonged period (up to two weeks) in the invaded cell. But it is unclear how the bacterium persists inside the cell (Garzoni and Kelley, 2009). Transcriptome analysis has suggested extensive modifications in bacterial gene expression post-invasion (Garzoni et al., 2007). Of note gene expression of metabolic genes, indicative of nutrient restricted conditions, see increased activity which is in contrast to several haemolytic toxins including *hla* (Garzoni et al., 2007). It is suggested that small colony variants (SCV), a phenotypic variant of some *S. aureus* strains, could be better adapted to the intracellular environment (Sendi and Proctor, 2009). SCVs produce less factors that might damage host cells, such as Hla, and have a reduced growth rate allowing these bacteria to persist in host cells for longer periods (Proctor et al., 2006). Once *S. aureus* has been taken up into host cells it can manipulate host cell apoptosis through EsxA and then can mediate release of the bacteria through the activity of EsxB (Korea et al., 2014).

The importance of host cell invasion by *S. aureus* is unclear but it is likely S. *aureus* invades cells to avoid the host immune response and establish a chronic infection (Sendi and

Proctor, 2009, Kalinka et al., 2014). It has also been shown that *S. aureus* internalisation was necessary to induce endothelial cell apoptosis (Haslinger-Loffler et al., 2005). This may contribute to entry or exit from the vasculature, which allows localised infections to become systemic (Clarke and Foster, 2006).

1.6 Superantigens

The SAgs are a family of non-glycosylated proteins secreted by pathogens which induce non-specific T-cell proliferation (Spaulding et al., 2013). SAgs bind MHC class II and the T-cell receptor (TCR) forming a trimeric complex which bypasses normal antigen presentation and activation (Fig 1.3) (Baker and Acharya, 2004, Fraser and Proft, 2008b). SAgs have been identified in a number of different bacterial species including *Streptococcus spp.*, *Yersinia enterocolitica* and *Mycoplasma arthritidis* in addition to some viruses such as Epstein-Barr virus and gammaherpes virus (Fraser and Proft, 2008b, O'Flaherty et al., 2015). The staphylococcal SAgs represent the best characterised group with over 25 identified to date (Table 1.3) (Ono et al., 2015, Spaulding et al., 2013). In general the primary function of SAgs is to subvert adaptive immune recognition and cause significant damage to the host, permitting the progression of infection (Stach et al., 2014). SAgs are generally heat and protease resistant and can also maintain functional activity after exposure to weak acids and weak bleach solutions, allowing the staphylococcal enterotoxins (SEs) to survive the harsh conditions of the gut and cause food poisoning (McCormick et al., 2001, Li et al., 2011)

Table 1.4 Properties of Characterised staphylococcal SAgs.

SAg	Molecular Mass (kDa)	Emetic[a]	Human Vβ specificity[b]	MHC class II binding[c]	Genomic location
SEA	27.1	+	1, 5, 6, 7, 9, 15, 16, 18, 21, 22, 24	LAS+HAS	ϕSa3n
SEB	28.3	+	3, 12, 13, 14, 15, 17, 20	LAS	SaPI
SEC	27.5	+	3, 12, 13, 14, 15, 17, 20	LAS	SaPI
SED	26.4	+	1, 3, 5, 8, 9, 12, 14	LAS+HAS	Plasmid (pIB485-like)
SEE	26.4	+	5, 6, 8, 9, 13, 16, 18, 21	LAS+HAS	Integrated Plasmid
SEG	27	+	3, 12, 13, 14, 15	LAS	vSAβ
SEH	25.2	+	Vα8, Vα10	LAS+HAS	Transposon
SEI	24.9	+	1, 5, 6, 23	LAS+HAS	Plasmid (pF5)
SEJ	28.5	+	8, 21	LAS+HAS	SaPI/ϕSa3n/Plasmid (pF5/ pIB485-like)
SEK	26	+	1, 5, 6	LAS+HAS	SaPI
SEL	26	+	1, 5, 7, 16, 22, 23	LAS+HAS	vSAβ
SEM	24.8	+	8, 9, 18, 21	LAS+HAS	vSAβ
SEN	26.1	+	7, 8, 9, 17	LAS+HAS	vSAβ
SEO	26.7	+	5, 7	LAS+HAS	vSAβ
SEP	27	+	5, 8, 16, 18, 21	LAS+HAS	ϕSa3n
SEQ	28	+	6, 21	LAS+HAS	SaPI/ϕSa3n
SER	27	+	3, 12, 14	LAS	Plasmid (pF5/ pIB485-like)
SES	26.2	+	9, 16	LAS+HAS	Plasmid (pF5)
SET	22.6	+	NK	LAS	Plasmid (pF5)
SElU	27.1	NK	13, 14	LAS	vSAβ
SElW	26.7	NK	NK	LAS	vSAβ
SElV	25	NK	6, 18, 21	LAS+HAS	vSAβ
SElX	19.3	NK	1, 6, 18, 21	NK	Core genome
SElY	22.5	+	NK	NK	NK
TSST-1	22	-	2	LAS	SaPI

[a] NK, Not known
[b] Data compiled from references: (Argudin et al., 2010, Spaulding et al., 2013, Omoe et al., 2013, Seo et al., 2010a, Ono et al., 2015)
[c] LAS, low affinity site α chain HAS, high affinity site βchain

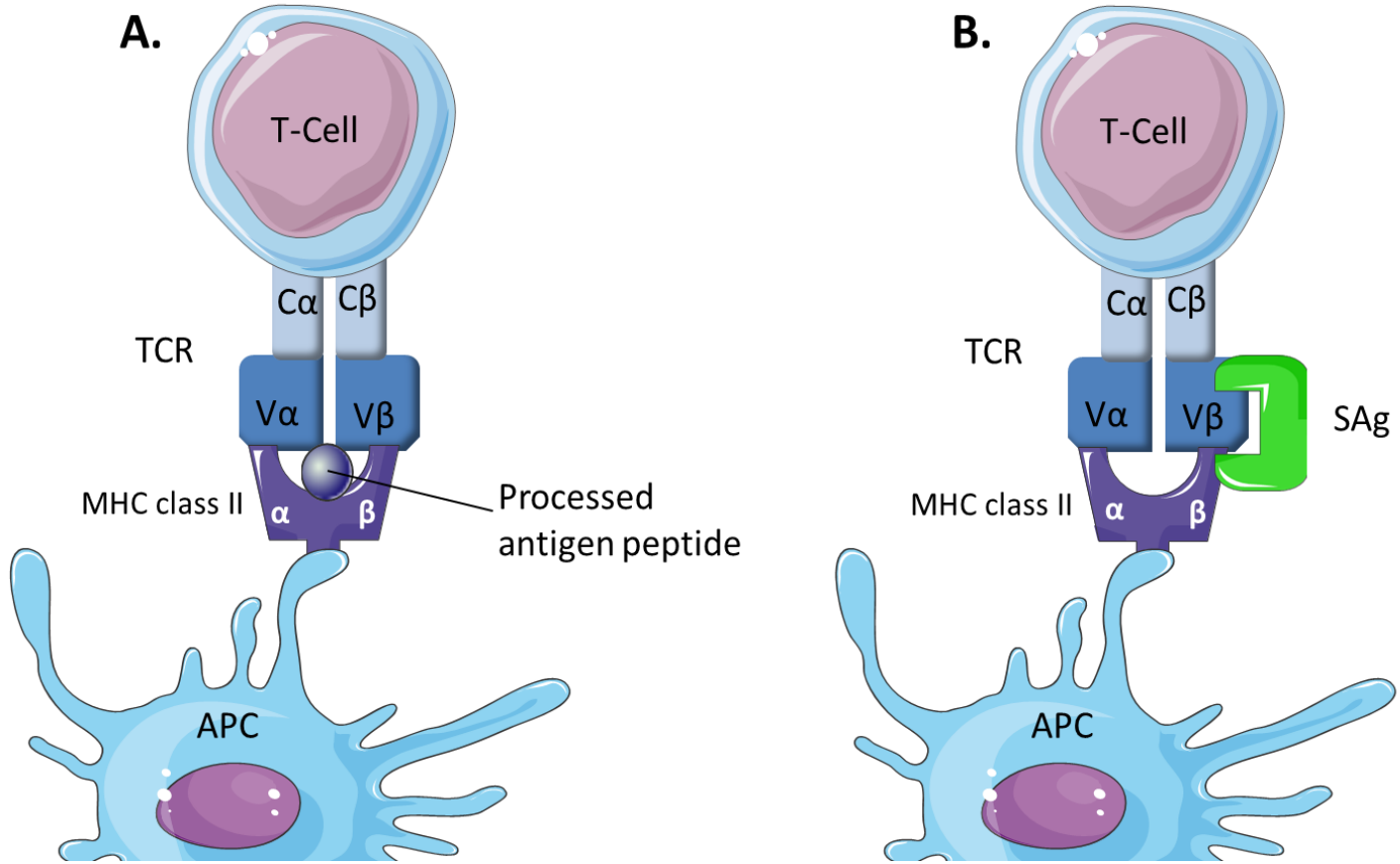
A.
T-Cell
Cα
Cβ
TCR
Vα
Vβ
MHC class II
α
β
Processed
antigen peptide
APC
B.
T-Cell
Cα
Cβ
TCR
Vα
Vβ
SAg
MHC class II
α
β
APC

Figure 1.3 Superantigens induce T-cell proliferation by by-passing normal antigen presentation. (A.) Conventional antigen presentation and specific T-cell activation. An antigen presenting cell (APC) processes antigen and loads an antigen peptide onto the binding site of the MHC class II molecule which in turn is presented to a specific T-cell receptor (TCR). (B.) SAgs crosslink the MHC class II and TCR which induces non-specific activation of T-cells. The SAg binds to the MHC class II outside the antigen presentation site and the Variable beta (Vβ) chain of the T-cell receptor.

1.6.1 SAg structure and binding

The staphylococcal SAg protein family ranges in size from 19 kDa to 29 kDa and they have two major domains including an N-terminal domain which displays a characteristic oligosaccharide/oligonucleotide binding (OB) fold, and a C-terminal domain which adopts a β-grasp motif (Fig 1.4) (Spaulding et al., 2013). These two domains are divided by a solvent accessible α-helix which spans the centre of the molecule (Fraser and Proft, 2008b). A feature seen in many SAgs is a highly flexible disulphide loop located in the N-terminal domain which is implicated in both the emetic activity of SEs and binding to certain TCR Vβ chains (Fig 1.4) (Hu and Nakane, 2014, Spaulding et al., 2013).

Another common feature among SAgs are zinc binding sites, which vary in arrangement and may be used as an alternative MHC class II binding site (Fig 1.4) (Li et al., 2001, Sundstrom et al., 1996). Zinc binding sites have also been shown to be important in the dimerization of staphylococcal enterotoxins such as SED or SEC (Chi et al., 2002, Sundström et al., 1996), and there is evidence to suggest that zinc binding may play a role in the high level of thermostability exhibited by many SAgs (Baker and Acharya, 2004, Cavallin et al., 2000a).

Staphylococcal SAgs also share a common dodecapeptide sequence which is located within β-strand(8)/hinge/α-helix(4) domain of SAgs and has been demonstrated to interact with epithelial cells and induce the production of cytokines, such as IL-8 (Shupp et al., 2002). This mechanism is termed outside-in signalling and it is proposed that it is exploited by SAgs to attract immune cells to the sub-mucosa upon which SAg can exert their activity (Brosnahan and Schlievert, 2011, Stach et al., 2014). Binding of co-receptors to the trimeric complex induced by SAgs has not been well characterised to date. However recent work conducted on SEB demonstrates this SAg not only binds the TCR and MHC but also the co-stimulatory receptor CD28 (Arad et al., 2011). The interaction between CD28 and SEB is

mediated through the SAg dodecapeptide implicated in outside-in signalling (Arad et al., 2011). Binding of this receptor was required to induce the release of pro-inflammatory cytokines suggesting that the mechanism of SAg-induced T-cell proliferation is not necessarily restricted to TCR-MHC interactions as previously assumed (Arad et al., 2011).

Two distinct sites have been identified on MHC class II that bind to SAgs. The first is referred to as the generic binding site and is located on the α chain of MHC-class II. The other type is located on the β-chain of the MHC class II and is termed the high affinity site, due to its ~100 fold greater affinity for SAg binding than the generic binding site. Binding at the high affinity site is mediated by zinc (Fig 1.4) (Baker and Acharya, 2004, Papageorgiou and Acharya, 1997, Papageorgiou and Acharya, 2000). The presence of multiple sites of interaction with MHC class II produces a great deal of diversity among these toxins as many SAgs, including TSST-1 and SEB bind to MHC class II molecules that express HLA-DR alleles while some other SAgs such as SEC, SpeA and SSA bind predominantly to HLA-DQ alleles (Jardetzky et al., 1994, Kim et al., 1994, Papageorgiou and Acharya, 2000, Patel et al., 2010).

Almost all SAgs interact with the Vβ chain of the TCR resulting in stimulation of up to 20% of resting T-cells (Spaulding et al., 2013). Highly specific interactions occurs between the active side chains in the SAg and complementarity determining regions 1 and 2 (CD1 and CD2) and hypervariable region 4 (HV4) of the TCR Vβ chain (Fields et al., 1996) and altering a single residue in the TCR binding site is enough to alter the Vβ-specificity (Li et al., 1998). Binding of the TCR by SAgs results in clustering of TCRs which in turn facilitates the acquirement of intracellular components required for signal transduction (Lavoie et al., 2001). This clustering may occur in two ways; firstly by direct clustering of the TCR or by the binding of SAg homodimers to multiple MHC class II molecules which would also promote TCR clustering (Fig 1.5) (Baker and Acharya, 2004, Lavoie et al.,

2001). Of note, SEH is the only staphylococcal SAg demonstrated to interact with the Vα chain of the TCR instead of the of the Vβ chain (Petersson et al., 2003, Saline et al., 2010).

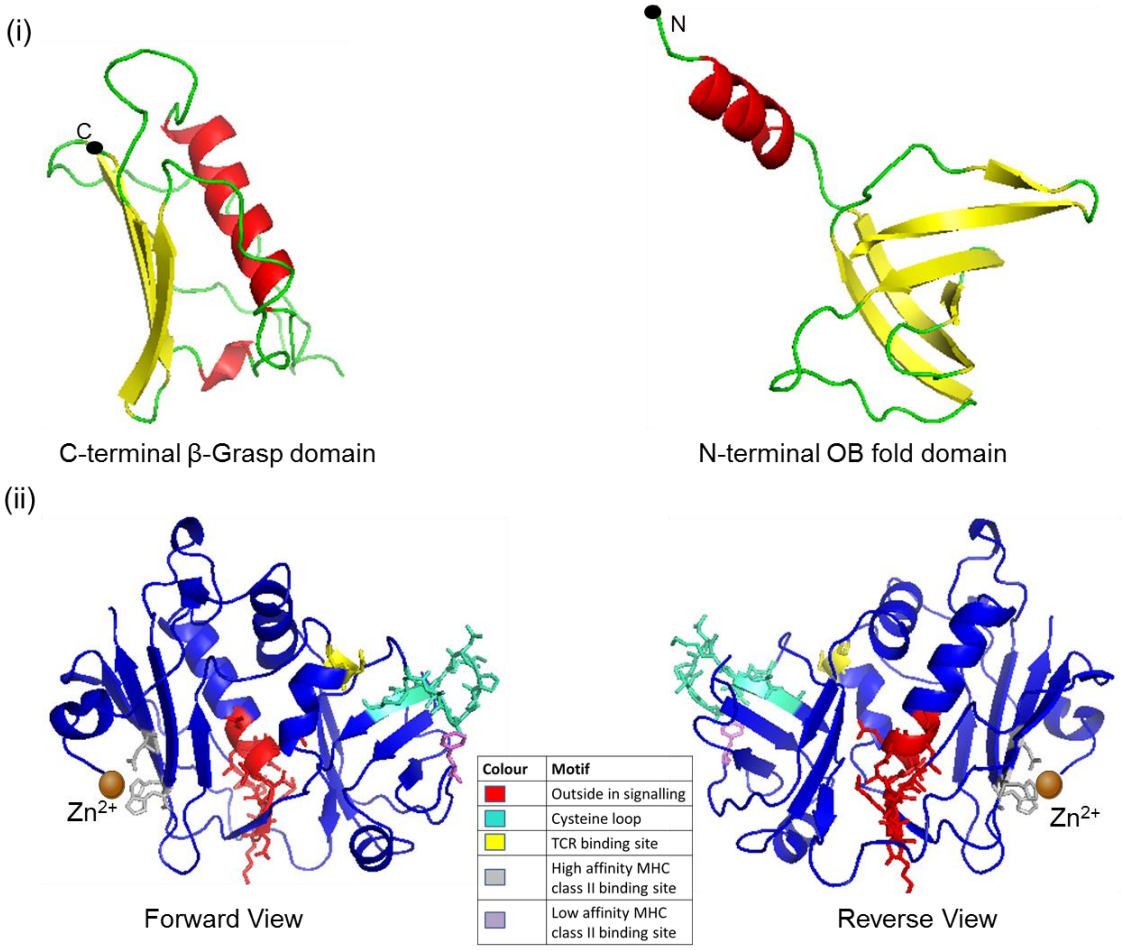

Figure 1.4 The two domain structure of SAgs. The staphylococcal SAg consists of two protein domains demonstrated by TSST-1 (i) . The crystal structrure of SEA reveals a number of features associated with SAgs (ii). The features are colured and residues involved are shown.

1.6.2 Role of SAgs in *S. aureus* pathogenesis

SAg induced T-cell proliferation elicits a response that is primarily inflammatory. CD4 and CD8 T- lymphocytes proliferate and a range of cytokines can be released including IL-1, IL-2, IL-6, tumour necrosis factor-α (TNF-α), IFN-γ, macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and monocyte chemo- attractant protein 1 (MCP-1) (Krakauer, 1999, Fraser and Proft, 2008b). This uncoordinated release of cytokines is the main cause of damage associated with SAg induced toxicity (Spaulding et al., 2013). SAgs can cause rashes, fever, multi-organ damage, coma and death from severe shock (Schlievert, 1995). These toxins have been implicated in a wide range of diseases ranging from staphylococcal food poisoning to severe toxic shock (Murray et al., 1995, Schlievert, 1995). SAgs have also been implicated in a range of autoimmune conditions including Kawasaki disease, multiple sclerosis, arthritis and diabetes (Torres et al., 2001, Stauffer et al., 2001).

SAg-induced T-cell proliferation leads to a pro-inflammatory response which causes damage to the host and impedes the effectiveness of the immune response by creating a bias towards the T_h1 response and inducing memory T-cell deletion or anergy (Watson et al., 2012). Prolonged exposure to SAgs results in the production of IL-4 and IL-10 and can lead to deletion or anergy of stimulated T-cells decreasing the repertoire available to interact with *S. aureus* infections (Miller et al., 1999, Watson et al., 2012). The production of IL4 and IL-10 act to decrease the immune response with affects such as a reduction in IFN-γ and depletion of MHC Class II which results in memory T-cells that are unresponsive to antigen stimulation (Miller et al., 1999, Sundstedt et al., 1997, Watson et al., 2012). Regulatory T-cells (T_{regs}) are also affected by SAg-induced T-cell proliferation and are unable to mitigate the inflammation caused by toxic shock syndrome (TSS) (Tilahun et al., 2014). SAgs have been shown to manipulate cytokine expression during infection and subvert the innate immune response by inducing the infiltration of neutrophils into murine livers forming abscesses via the induction of cytokine release (Xu et al., 2014). Taken together the effects

induced by SAgs are likely to cause significant deficiency in the ability of the adaptive immune response to respond effectively to infection by the uncontrolled release of cytokines leading to global immune dysregulation.

SAgs can also play a role in disrupting mucosal barriers and mediating immune inflammation at these locations (Brosnahan and Schlievert, 2011). The outside-in signalling dodecapeptide motif has been implicated in this mechanism as it has been shown to mediate transcytosis of SEA, SEB and TSST-1 through epithelial monolayers (Shupp et al., 2002). The mechanism proposed is that SAgs can interact directly with epithelial cells and stimulate the release of chemokines such as IL-8 and MIP-3α which recruits immune cells to the submucosa and the lumen, particularly neutrophils and T-lymphocytes (Peterson et al., 2005). The influx of neutrophils into the lumen can result in severe pathology as observed in a murine pneumonia model of intranasal exposure to SEB (Rajagopalan et al., 2006). The influx of immune cells results in disruption of the epithelial barrier allowing the SAg to diffuse into the submucosa (Rajagopalan et al., 2007). At this point the SAg can interact with T-lymphocytes, both in the submucosa and with lymphocytes that have migrated thorough the epithelium, and induce further inflammation (Spiekermann and Nagler-Anderson, 1998). The stimulation of the mucosal surface by SAgs results in inflammation and barrier disruption which can lead to invasion by *S. aureus* and in some cases allows the SAg to enter the blood stream resulting in systemic toxic shock (Brosnahan and Schlievert, 2011).

S. aureus is a major cause of the food poisoning in humans and this is characterised by the rapid onset of vomiting and is mediated by the emetic SAgs (Table 1.4) (Hu and Nakane, 2014, Hennekinne et al., 2012). The first staphylococcal SAgs to be described were SEA and SEB, which were initially identified as enterotoxins before the superantigenic function of the protein was elucidated (Balaban and Rasooly, 2000). SEA is still the most common SE associated with staphylococcal food poisoning and much of the research to

understand the mechanisms of emesis induced by SEs have been performed with SEA (Hu and Nakane, 2014). SEA can translocate from the gastrointestinal tract (GI) lumen through the intestinal epithelium into the submucosa possibly using the same outside-in signalling mechanism observed for TSST-1 (Hu et al., 1999, Brosnahan and Schlievert, 2011). It has been shown that SEA can interact with mast cells in the submucosa and induce 5-HT serotonin release by the degranulation of mast cells (Ono et al., 2012). The release of 5-HT serotonin can stimulate vagal afferent nerves in the submucosa by binding the 5-HT_3 receptors (Hu et al., 2007). The binding of 5-HT serotonin to vagal nerves can induce nerve depolarisation which can stimulate the medulla oblongata leading to the vomiting reflex (Darmani and Ray, 2009). Emesis induced by enterotoxins occurs rapidly, these toxins can enter and progress through the GI tract independently of *S. aureus* bacteria owing to the high level of structural stability exhibited by SEs (Hennekinne et al., 2012, Li et al., 2011).

SAgs have been shown to be important in the onset of necrotizing pneumonia most likely by stimulating infiltrating $CD4^+$ T-cells inducing proliferation and the production of pro-inflammatory cytokines including IL-6, IL-1β and TNF resulting in tissue damage (Parker et al., 2015, Strandberg et al., 2010). SAgs has been shown to be critical in the establishment of IE as TSST-1- negative isogenic isolates of a USA200 clone were unable to establish IE in rabbits unlike TSST-1 positive isolates that were able to establish severe IE at a much higher frequency (Pragman et al., 2004). Anti-SEC therapy also reduced the severity of disease in a rabbit IE model (Mattis et al., 2013). In this disease the role of SAgs is unclear but there is evidence to suggest a number of mechanisms including direct toxicity of the endothelium cells, immune dysregulation preventing clearance of the vegetation or capillary leakage induced SAgs all could be responsible (Lee et al., 1991b, Lee et al., 1991a, Spaulding et al., 2013).

1.6.3 Distribution of *S. aureus* SAg Genes

The distribution of SAgs in *S. aureus* is highly variable and each strain can secrete anywhere between one and 23 of 25 serologically distinct SAgs (Spaulding et al., 2013). 80 % of human nasal isolates were found to encode at least one SAg (in addition to SElX), and of this number 50% were found to have the enterotoxin gene cluster (*egc*) suggesting the majority of human clinical isolates encode on average 5 SAg genes (Omoe et al., 2005). The prevalence of SAg genes is higher in MRSA than in MSSA as the SCC*mec* element can contain SAg genes (Hu et al., 2011, Hu et al., 2008). The diversity of SAg carriage results largely from of almost all SAg genes being associated with MGEs such as prophages, plasmids and SaPIs (Table 1.4) (Argudin et al., 2010). SaPIs can range in size from 14 kbp to17 kbp and can encode a variety of different SAgs depending on the SaPI (Suzuki et al., 2015, Argudin et al., 2010). The mobility of these elements are mediated by phage, for example, SaPI2 containing the *tst*-1 gene is excised from its chromosomal location and transmitted during the growth of the phage 80α (Subedi et al., 2007, Ubeda et al., 2009). The enterotoxins SED and SEJ are both found associated with the same plasmid termed *sed*-encoding plasmid (Zhang et al., 1998). SEA is encoded on the IEC, which includes the genes for the immune modulators SCIN and CHIPS and the IEC is transferred by β-haemolysin converting phage (van Wamel et al., 2006). The enterotoxin genes *seg*, *sei*, *selm*, *seln*, and the two pseudogenes *ψent*1 and *ψent*2 have been demonstrated to form the chromosomal operon *egc* (enterotoxin gene cluster) which is located within the vSaβ genomic island (Jarraud et al., 2001b). The *egc* of *S. aureus* appears to have been formed through the duplication and diversification of a SAg ancestor, and phylogenetic analysis of this cluster and other SAg genes in *S. aureus* suggested that all these genes are related (Jarraud et al., 2001b). It has been demonstrated that the vSaβ element was transferred by multiple overlapping bacteriophage transduction resulting in the diversification of this element in different *S. aureus* strains (Moon et al., 2015). This mechanism may also explain how newly

diversified SAg were transferred from the *egc* nursery to other parts of the *S. aureus* genome. The presence of the *egc* cluster can enhance the fitness of the bacteria and is found in many clinical isolates of *S. aureus*, although animal studies have suggested that this cluster may play more of a role in colonisation than infection (Nowrouzian et al., 2015).

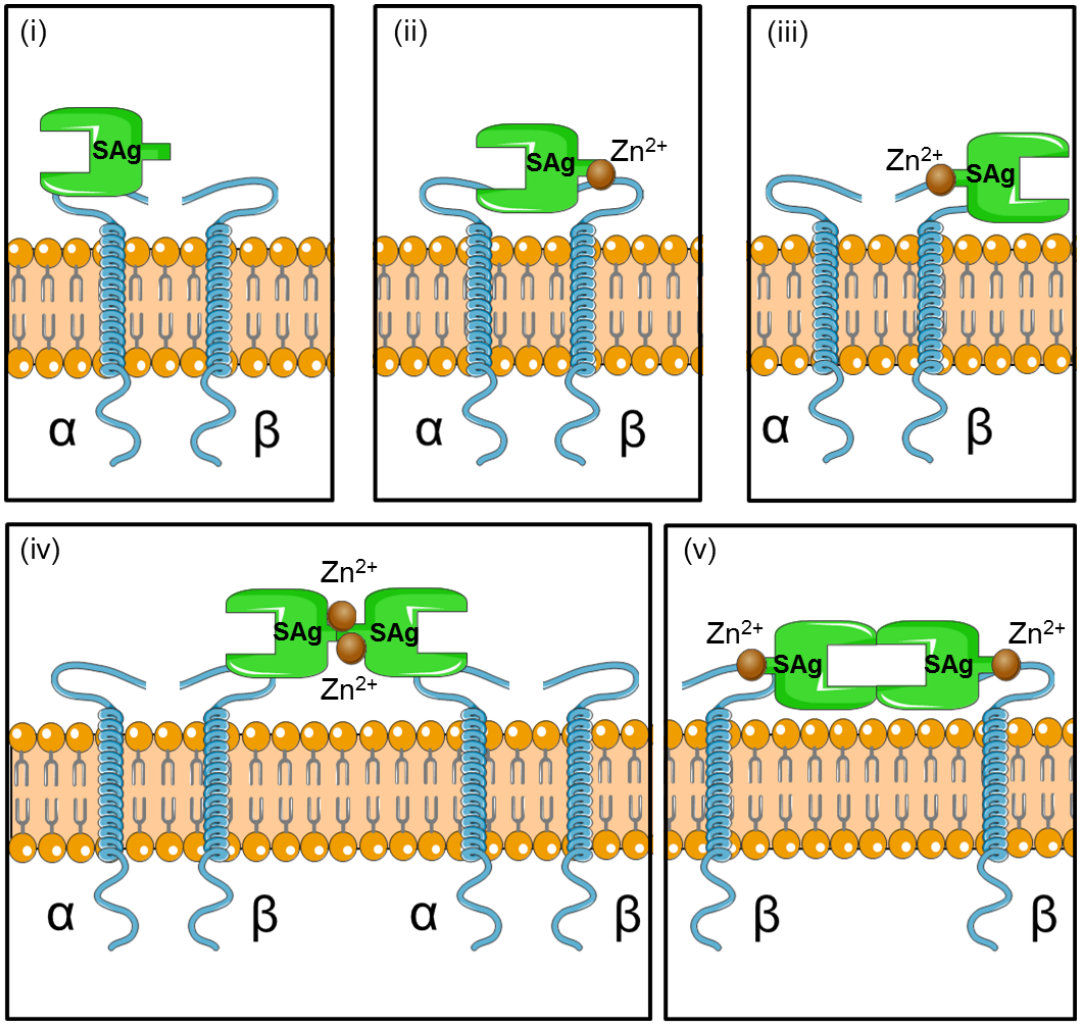

Figure 1.5: Different SAgs can bind to the MHC class II molecule through a variety of mechanisms. Schematic diagram of how Superantigens (SAgs) can interact with the MHC class II molecule (blue, made up of both an α-chain and β-chain) (brown sphere represent Zn^{2+} ion); (i) Zinc independent binding through low affinity site on α chain (e.g. TSST-1). **(**ii) Zinc dependent binding to the β chain through the high affinity site (e.g. SEI). (iii) Binding to both sites (e.g. SEA). (iv) Dimerization of SAgs via zinc binding (e.g. SED). (v) Bind to MHC class II via high affinity site allowing dimerization via a different site (e.g. SPEC).

1.7 Regulation of virulence determinants in *S. aureus*

The coordinated regulation of virulence factors is essential to be able to establish an infection and evade the host immune system. To regulate gene expression, *S. aureus* utilises two major groups of regulators, transcription factors and two-component regulation systems (of which *S. aureus* has at least 16) (Table 1.5)(Cheung et al., 2004) These systems allow *S. aureus* to respond effectively to different environmental and host factors and act as an efficient opportunistic pathogen (Cheung et al., 2004). For example *sae* and *agr* deletion mutants of highly pathogenic USA300 strains are less virulent in a mouse model of necrotizing pneumonia and skin infection (Montgomery et al., 2010). These same regulators have been shown to be activated during interactions with neutrophils (Wright et al., 2005, Voyich et al., 2009).

The *agr* locus is largely responsible for growth phase dependent gene expression in *S. aureus.* It is a quorum sensing system which responds to the concentration of an auto-inducing peptide (AIP) (Painter et al., 2014). The locus is divided into two RNA transcripts, RNAII contains the operon *agrBDCA* and RNAIII is a regulatory RNA which acts as the effector of the *agr* cascade (Painter et al., 2014). The *agr* locus is a critical regulator for a large number of virulence determinants, up-regulating secreted factors such as *hla* and down-regulating cell associated determinants such as *spa* (Bronner et al., 2004, Kong et al., 2006). *Agr* can be activated during uptake into the phagosome by neutrophils, as the concentration of the auto-inducing peptide is higher in the lower volume of the phagosome. This can lead to the production of proteins that aid intracellular survival such as PSMs and Hla (Pang et al., 2010, Surewaard et al., 2013).

Table 1.5 Characterised two-component regulators of *S. aureus*.

Regulator	Activation	Function	Reference
AgrCA	Auto-inducing peptide (AIP)	Growth Phase dependent expression of virulence determinants	(Painter et al., 2014)
SaeRS	Stimuli from phagocytes	Regulates the expression of virulence determinants in response to phagocytic signals and elements of the humoral response. SaeRS can also work synergistically with sarA to repress proteolytic activity to promote biofilm formation.	(Mrak et al., 2012, Liang et al., 2006, Voyich et al., 2009, Geiger et al., 2008)
lytRS	Unknown	Regulates autolytic pathways in the bacterial cell which play a role in cell wall turnover and biofilm formation	(Brunskill and Bayles, 1996, Ingavale et al., 2003, Thomas and Hancock, 2009)
SrrAB	Oxygen starvation	Down-regulates virulence determinants under anaerobic conditions. Up-regulates genes involved in nitric oxide resistance.	(Kinkel et al., 2013, Ulrich et al., 2007)
HssRS	Heme	System is activated by heme resulting in the up-regulation of the ABC transporter HrtAB which mediates heme uptake.	(Stauff and Skaar, 2009)
WalKR	Unknown	Regulation of cell wall maintenance, autolysis genes and biofilm formation.	(Dubrac et al., 2007)
AirSR	Anoxic conditions	Regulates virulence expression under anoxic conditions and can promote *S. aureus* survival in blood. Regulates cell homeostasis.	(Hall et al., 2015, Sun et al., 2012)
ArlRS	Unknown	Regulation of autolysis and proteolytic enzyme release along with regulation of agglutination.	(Walker et al., 2013, Fournier and Hooper, 2000)
BraSR/ NsaSR	Antibiotics, bacteriocins	Regulation of resistance to both antibiotics and bactreiocins by the activation of a corresponding ABC transporter that mediates detoxification	(Hiron et al., 2011, Yoshida et al., 2011)
GraRS	Antimicrobial peptides	Activates the expression of the vraFG ABC transporter and confers resistance to some cationic antimicrobial peptides	(Yang et al., 2012)
HptRS	Hexose phosphates	Mediates the expression of the system required for uptake of hexose as a carbon source	(Park et al., 2015)
VraRS	Antimicrobials	Contributes to resistance against β-lactams such as oxicillin but independent of *mecA* regulation	(Boyle-Vavra et al., 2006)

The *sae* locus is a two-component system critical for the optimal expression of exoproteins and is made up of four open reading frames (ORF), *saeS, saeR, saeQ* and *saeP* (Cheung et al., 2004). The ORFs *saeS* and *saeR* encode the two-component system; where *saeS* encodes a histidine kinase and *saeR* encodes the cytoplasmic response regulator (Giraudo et al., 1999). The presence of *saeQ* and *saeP* has been demonstrated to be essential for this locus to function correctly through the enhancement of phosphatase activity by the SaeS sensor kinase (Adhikari and Novick, 2008, Jeong et al., 2012). The expression of SaeQ and SaeP can lead to differential expression of SaeR targets (Adhikari and Novick, 2008, Jeong et al., 2012). In USA300 strain LAC, SaeR up-regulates virulence-associated genes sharing a consensus sequence found in the promoter regions (Nygaard et al., 2010). The SaeRS system is critical for the regulation of factors involved in innate immune evasion and if deleted *S. aureus* virulence is highly attenuated (Voyich et al., 2009, Montgomery et al., 2010). SaeRS has been shown to be activated by signals indicative of phagocytes including hydrogen peroxide and low concentrations of α-defensins (Geiger et al., 2008). Examples of immune evasion factors regulated by SaeRS include the SSl-proteins which are upregulated when SaeRS is activated together with Rot (Repressor of toxins) (Benson et al., 2012). SaeRS can also coordinate with the post-transcriptional repressor SarA and repress the expression of proteolytic enzymes allowing the formation of *S. aureus* biofilm (Mrak et al., 2012).

Rot is a post-transcriptional repressor, which is a member of the SarA family. It has been shown to repress the α-toxin gene, *hla,* along with a large group of other virulence factors including SAgs genes like *seb* (Manna and Ray, 2007, Tseng and Stewart, 2005) . In addition to its role as a toxin repressor Rot has been shown to be a global regulator of virulence that can promote expression of genes encoding SSl-proteins (Benson et al., 2011, Said-Salim et al., 2003). The activity of Rot is required for biofilm formation of USA300

strains by supressing the activity of proteases secreted during the planktonic phase (Mootz et al., 2015).

Global regulators do not operate independently; the *saeRS* system coordinates with the *agr* locus, combining environmental and quorum sensing signals to regulate virulence expression (Fig 1.6). An example of this interaction between global regulators is the expression of *hla*, which has been shown to be co-ordinated by a combination of factors including *agr*, *saeRS* and *rot* (Xiong et al., 2006). A further example of a virulence factor regulated by a combination of different global virulence regulators working in tandem is TSST-1 which in addition to being regulated by RNAIII also has been shown to be under the influence of sigma factor B, Rot and SarA (Andrey et al., 2015). The complex regulation of virulence determinants allows for differential expression of virulence factors so they can be produced at the optimal time during infection.

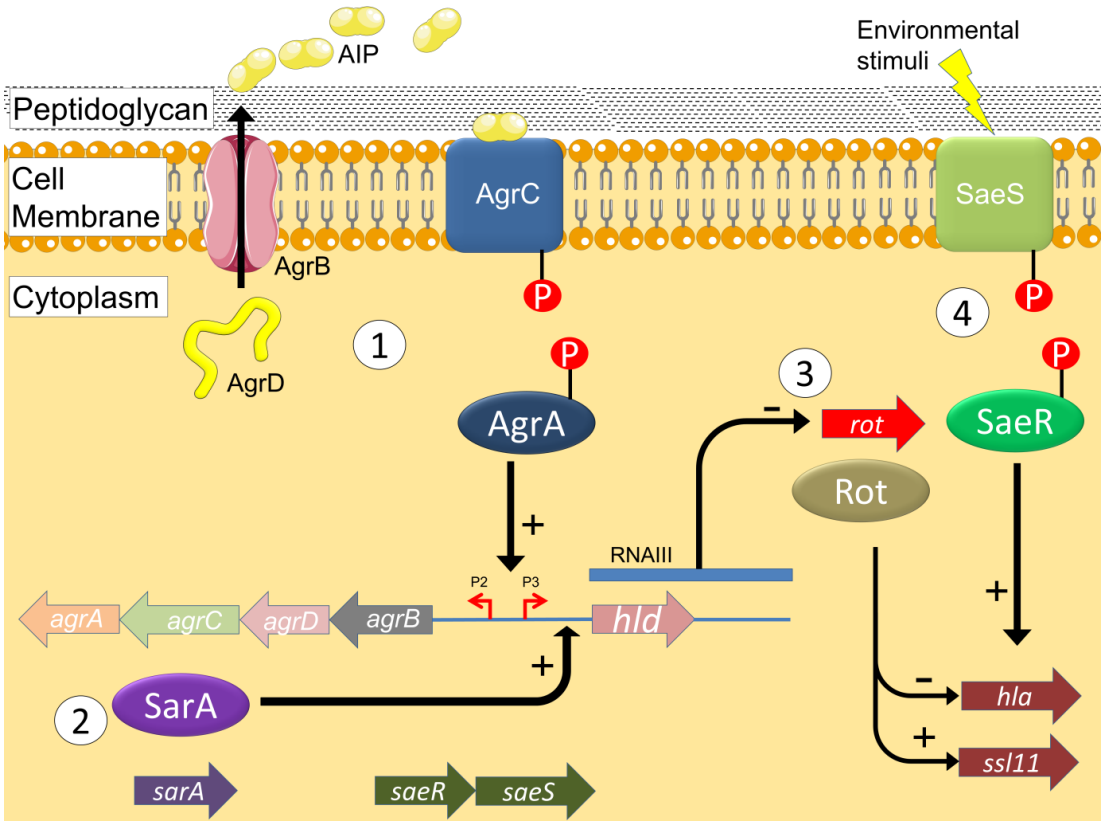

Figure 1.6: *S. aureus* virulence regulators coordinate to ensure correct expression of virulence determinants. Major regulatory inputs in the expression of *hla* and *ssl11* are shown. (1) The Agr quorum-sensing system is activated when AIP reaches a high concentration as a result of high bacterial density, leading to AgrA-dependent activation of the P3 promoter, which encodes the regulatory RNA, RNAIII. RNAIII negatively regulates the translation of the *hla* repressor and ssl11 activator Rot, leading to increased HlA production and reduced SSl11production. (2) The global regulator SarA indirectly facilitates expression by positively regulating the expression of the P3 promoter in the *agr* locus, leading to a similar repression of Rot translation and increased Hla production. (3) Rot is believed to bind directly to promoters to inhibit toxin gene expression. (4) The SaeRS two-component system recognizes external stimuli from the environment, leading to direct binding of SaeR to *hla* and *ssl11* promoters and subsequent enhancement of gene expression. This figure was adapted from Alonzo and Torres (2014).

1.8 Staphylococcal enterotoxin-like toxin X (SElX)

SElX is a SAg encoded in the core genome of 95% of *S. aureus* strains (Wilson et al., 2011). This is unique as all other SAgs characterised from *S. aureus* to date are associated with MGEs. SElX has demonstrated the typical properties of SAgs including, Vβ-specific T-cell mitogenicity, pyrogenicity and enhancement of LPS toxicity (Wilson et al., 2011). Sequence analysis of *selx* revealed 17 different allelic variants of this toxin, which have been generated by a combination of point mutation and assortative recombination (Wilson et al., 2011). Western blot analysis of human, bovine and ovine serum from clinical cases and human volunteers found that the majority of serum samples were positive for anti-SElX antibodies. This suggests that SElX is expressed during *S. aureus* infection and induces a humoral immune response (Wilson et al., 2011). Importantly SElX from the USA300 clone was shown to contribute to lethality in a rabbit model of necrotising pneumonia (Wilson et al., 2011). Global transcription studies of USA300 strain LAC have demonstrated that *selx* is up regulated when *S. aureus* is incubated in blood and serum unlike the other SAgs encoded by USA300 LAC and *selx* is expressed at similar levels to immune evasion proteins such as the *ssls* (Malachowa et al., 2011). Further study examining temporal expression of *S. aureus* virulence determinants during soft tissue infection in rabbits demonstrated SElX was regulated 1 day post infection in a similar fashion to known virulence factors such as PVL and Hla (Malachowa et al., 2015). The promoter region of *selx* contains a consensus sequence that mediates binding of SaeR and suggests that *selx* is regulated by this important 2-component regulator. SElX can interact directly with the surface of neutrophils which was demonstrated recently using a phage display approach (Fevre et al., 2014). The neutrophil interaction was shown to be of high affinity and could be abolished if the neutrophils were treated with neuraminidase suggesting the interaction was dependent upon sialic acid residues on the neutrophil surface (Fevre et al., 2014). An antibody blocking screen suggested the PSGL-1 can interact with SElX although a very high level of SElX protein was

required to see this (Fevre et al., 2014). It is likely given that the discrepancy of between SElX affinity for neutrophils and its relatively low affinity for PSGL-1, there is one or more targets for SElX still to be identified, along with any functional consequences for the neutrophil when SElX binds to it.

1.9 Staphylococcal enterotoxin-like toxins Y and Z (SElY and SElZ)

The toxin SElY was recently reported and shown to be able to induce emesis in the house musk shrew model and induce proliferation of human lymphocytes (Ono et al., 2015). The protein is predicted to have a molecular weight of 22.5 kDa and the amino acid sequence has a 32% sequence identity with the superantigen SET (Ono et al., 2015). SElZ is a putative SAg identified by Wilson (2010) in the bovine mastitis strain of RF122, which shares a 98% nucleotide identity with a putative SAg found in the capsule type 1 element SCCcap1 in *S. aureus* strain M. The closest related SAg is SEG which shares a 58% homology with SElZ (Wilson, 2010). Recombinant SElZ prepared from RF122 was able to induce bovine T-cell mitogenicity and the T-cells had a specific Vβ-activation profile in bovine T-lymphocytes (Wilson, 2010).

1.10 Hypothesis

The SAgs SElX, SElY and SElZ have all been recently identified and further examination of these SAgs is required to enhance our understanding of the role these proteins play in *S. aureus* pathogenesis. SAgs are produced by all clinical isolates and can modulate the host immune response by inducing T-cell activation, inducing damage through uncontrolled cytokine release and the induction T-cell anergy all contributing to pathogenesis. The SAgs secreted by *S. aureus* are highly diverse yet functionally very similar and as a result SAgs

may overlap in the T-cell groups that they activate. The SAgs are a large group of proteins probably reflecting the diversity of interactions with the adaptive immune response. However the energy cost of producing a group of virulence factors that have related immune modulatory functions is presumably high and it is therefore feasible that SAgs exhibit other functions to mitigate the energy costs. Given the structural relatedness of SAgs and SSls, it is possible that some SAgs have evolved to exhibit functions related to those of the SSl-proteins. SElX is a good candidate for exploring this further as it is one of the most closely related SAgs to the SSl-proteins and it has already been demonstrated to interact with PSGL-1 on the surface of neutrophils (Wilson et al., 2011, Fevre et al., 2014). The functional consequences of this interaction are yet to be investigated but may provide new insights into the interaction between SAgs and leukocytes within the immune system.

1.10 Project aims

The aim of this project is to investigate the functions of the staphylococcal SAgs SElX, SElY and SElZ and further elucidate the role of SAgs in *S. aureus* disease by:

- Investigating the structure of SElX, SElY and SElZ to inform our understanding of function
- Defining the emetic potential of SElX, SElY and SElZ to determine if these toxins can play a role in staphylococcal food poisoning
- Studying the relationship between SElX, SElY and SElZ with the innate immune system

Chapter 2

General Materials and Methods

2.1 Human blood sampling ethical review

For the isolation of human leukocytes, blood was sampled from healthy donors recruited by passive advertising. Written informed consent was obtained from all subjects and blood was obtained by venepuncture. Recruitment and sampling procedures were reviewed by the national research ethics service (NRES) committee London City and East under the research ethics committee reference 13/LO/1537. Details of the project are published on the following website: http://www.hra.nhs.uk/news/research-summaries/responses-of-human-immune-cells-to-bacterial-infection/

2.2 Bacterial culture conditions

S. aureus strains were grown in tryptone soya broth (TSB) or brain heat infusion broth (BHI) (Oxoid, UK) with shaking at 200rpm, or on tryptone soya agar (TSA) (Oxoid, UK) at 37°C for 16 h unless otherwise stated. *E. coli* strains were grown in Luria broth (LB) (Melford laboratories, UK) with shaking at 200rpm, or on LB-agar (Melford laboratories, UK) at 37°C for 16 h unless otherwise stated. Media was supplemented, where appropriate, with antibiotics. Strains were stored in appropriate liquid culture media containing 40 % (v/v) glycerol (Sigma-Aldrich, UK) in cryovials (Nunc, Thermo Scientific, UK) at -80°C.

2.3 Genomic DNA extraction

Genomic DNA (gDNA) was extracted from 0.5 ml of an overnight culture of *S. aureus* strains using the PurElute™ Bacterial Genomic Kit (Edge Biosystems, MD, USA) as described in the manufacturer's protocol, except for the addition of 100 µg/ml of lysostaphin (AMBI products LLC, NY, USA) to spheroplast lysis buffer and incubation for 30 min at 37°C. gDNA pellets were re-suspended in 50µl of EB buffer (Qiagen, Manchester, UK).

2.4 Plasmid and DNA isolation

Plasmids were isolated from 5 ml overnight cultures of *E. coli* using the QIAprep Spin Miniprep kit (Qiagen, Manchester, UK) as described in the manufactures protocol. DNA fragments were purified from agarose gels using the QIAquick gel extraction kit (Qiagen, Manchester, UK) according to the manufacturer's specifications. PCR products were cleaned up using the QIAquick PCR purification kit (Qiagen, Manchester, UK) according to the manufactures protocol.

2.5 DNA quantification

DNA concentration (ng/µl) was determined using a nano-drop ND-1000 spectrophotometer (Thermo scientific, USA) by measuring the absorbance at a wavelength at 260nm. Sample purity was assessed by comparing the ratios at wavelengths of 230/260 and 280/260.

2.6 Restriction digestion

Plasmid DNA was digested with appropriate high fidelity restriction enzymes and buffers as outlined in the manufacturer's protocol (New England Biolabs, Herts, UK). 50 ng of plasmid DNA was digested in a 20µl reaction containing 1x reaction buffer, 1U restriction endonuclease and ddH_2O for 1 h at 37°C.

2.7 Polymerase chain reaction (PCR)

Oligonucleotide primers were designed using the primer design tool on the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and synthesised by Invitrogen custom oligo service (Life Technologies, Paisley, UK). PCR reactions were carried out in Mycycler™ thermal cycler (Biorad, UK) and contained 100 nM forward and reverse primers and 250 nM dNTP (Promega, Hampshire, UK), either Go Taq G2 polymerase enzyme (Promega, Hampshire, UK) or PfuUltra II Fusion DNA polymerase (Agilent technologies, UK) at 1 U per reaction, PCR buffers for each enzyme were provided with the kit and used at a 1 x concentration. 10 ng of template DNA was added and ddH_2O was added to a final volume of 50 µl. Colony PCR was carried out by boiling single colonies for 10 min in 20 µl of ddH_2O. Unless otherwise stated, the thermal programme was; 1 cycle at 95°C for 3 min, 30 cycles at 95°C for 1 min, 54°C for 1 min, 72°C for 1 min per kb (Taq) or 15 s per kb (Pfu), followed by a final extension of 72°C for 10 min.

2.8 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA fragments was carried out in 1 % (w/v) agarose gels (Melford Laboratories, UK), with 1 x Tris-acetate-EDTA (TAE) buffer (Thermo Scientific, UK) containing either 1 x SYBR safe DNA gel stain (Life Technologies, Paisley, UK) or 1 x Gel Red DNA stain (Biotium Limited, Cambridge Biosciences, UK). Promega loading dye (Promega, UK) was added to DNA samples before they were loaded onto the gel alongside a 1 kb DNA ladder (Promega, UK). Electrophoresis was carried out at 120 V for 60 min. Gels were visualised using the UV transilluminator function on a G-box (Syngene, Cambridge, UK).

2.9 DNA sequencing

Sequencing reactions were carried out by Edinburgh Genomics (King's Buildings, University of Edinburgh, UK). Reactions contained 5 µl (approximately 50 ng) of purified DNA with 100 nM sequencing primers. Big Dye terminator reactions were performed as part of the sequencing services using the Bigdye™ Terminator v3.1 Ready Cycle reaction sequencing kit and performed according to the manufacturer's protocol (Life Technologies, UK). Samples were analysed on an ABI3730 capillary DNA analyser (Life Technologies, Paisley, UK).

2.10 Gene Cloning

Gene templates were amplified by PCR using PfuUltra II Fusion DNA polymerase (Agilent technologies, UK) with primers stated in each chapter. PCR products were checked by gel electrophoresis to ensure robust amplification and then the PCR inserts were used in the Strataclone blunt cloning kit (Aglient technologies, UK). The PCR insert was cloned into the pSC-B plasmid as described in the manufacturer's protocol. Following cloning the pSC-B plasmids were isolated using the QIAprep minikit (Qiagen, Manchester, UK) and the purified plasmid was then digested with restriction enzymes. The inserts were purified from a gel and ligated into the desired plasmid (digested with the same enzymes as the inserts) with T4 DNA ligase (New England Biolabs, Herts, UK) for 16 h at 16°C. The ligations were dialysed on 0.025 µm filter discs (Merk Millipore, Germany) and transformed into *E. coli* strains DH5α or XL-1 blue. For the plasmids transformed into DH5α cells, the plasmids were subsequently isolated and transformed by electroporation into *E. coli* BL21 DE3 for expression.

2.11 Preparation of electo-competent cells and electroporation

E. coli electro-competent cells were prepared by inoculating 250 µl of an overnight culture into 25 ml of LB and incubated at 37°C with shaking at 200 rpm, until an OD_{600} of 0.6 was reached. Cells were washed once in an equal volume of ddH_2O, after centrifugation at 4000 x g for 10 min, at 4°C. The cells were then washed twice in a ½ volume of ice cold 10 % (v/v) glycerol and once in ¼ volume of ice cold of 10 % glycerol. Cells were pelleted as before and re-suspended in 250 µl of 10 % glycerol. Electro-transformation was carried out with approximately 1 µg of plasmid added to 50 µl of competent cells in an electroporation cuvette with a 0.2 cm gap (Sigma-Aldrich, UK). Electroporation was carried out at 100 Ω, 25 µF and 2.5 kV and immediately following pulsing 250 µl of super optimal broth (SOB) was added and the suspension was then incubated at 37°C for 1 h with shaking at 200 rpm. Cells were plated onto LB-agar supplemented with selective antibiotics and incubated for 18 h at 37°C.

2.12 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were resolved by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). 12 % (w/v) polyacrylamide resolving proteins gels were prepared using a mini-protean casting apparatus (Biorad, Hemel Hempstead, UK). The polyacrylamide gel was prepared with 4 ml of ddH_2O, 2.5 ml of 1.5M TrisHCl (pH 8.8), 100 µl 10 % (w/v) Sodium-dodecylsulphate (SDS) (Melford, UK), 3.3 ml 40 % (w/v) Acrylamide/Bis-acrylamide (Sigma-Aldrich, UK), 100 µl 10 % (w/v) Ammonium persulphate (APS) (Sigma-Aldrich, UK) and 10 µl Tetramethylethylenediamine (TEMED) (Sigma-Aldrich, UK) and sealed using 1 ml isopropanol. The gel was allowed to polymerise for at least 30 min before

a 4% (w/v) stacking gel was added which contained 6.30 ml of ddH_2O, 2.5 ml of 0.5 M TrisHCl (pH 6.8), 100 µl 10% (w/v) SDS, 1 ml 40% (w/v) Acrylamide/Bis-acrylamide, 100 µl 10% (w/v) APS and 10 µl TEMED. A comb was inserted once the stacking gel was added and the gel was allowed to polymerise. The protein gel was then assembled in the mini-protean tetra-cell running apparatus (Biorad, Hemel Hempstead, UK) and covered with 1 x running buffer (5 x stock containing; 124 mM Tris hydroxymethyl (methylamine) base (Tris) (Fisher Scientific, UK) 960 mM glycine (Sigma-Aldrich, UK) and 17 mM SDS). Protein samples were boiled in 1 x protein sample buffer (Laemmli, Sigma-Aldrich, UK) for 10 min, loaded into wells and electrophoresed at 200 V for 40 min. A pre-stained Pageruler™ 180kDa to 10 kDa (Life Technologies, UK) molecular marker was included for protein size estimation. SDS-PAGE gels were stained for 1 h at room temperature with Instant Blue coomassie stain (Expedeon, Cambridge, UK).

2.13 Recombinant protein purification

2.13.1 Protein induction

E. coli expression strains containing plasmid constructs for protein expression were cultured in LB containing selective antibiotics and induced in mid-exponential phase of growth (OD_{600} = 0.6), with 1 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) (Formedium Ltd., Norfolk, UK) for 4 h at 37°C or 18 h at 16°C. Once induced, cells were harvested by pelleting the cells at 4000 x g for 20 min and stored at -20°C if not processed immediately.

2.13.2 Affinity chromatography

Recovered cells were defrosted and re-suspended in binding buffer (either native or denaturing buffer depending on the protein) (Table 2.1), before lysis in a One-Shot cell disruptor (Constant Systems, Northants, UK). 6 x HIS-tagged recombinant proteins were

purified by automated immobilized metal affinity chromatography (IMAC) with Ni-NTA superflow resin (Qiagen, Manchester, UK) along with employing the buffers listed in Table 2.1. Purification was performed on an AKTA fast protein liquid chromatography (FPLC) OPC900 P920 machine (GE Healthcare, Buckinghamshire, UK). If a second purification step was required ion exchange chromatography protein purification was also performed using an SP sepharose column (GE Healthcare, Buckinghamshire, UK) (Binding and wash buffer 50mM HEPES and elution buffer 50mM HEPES+1M NaCl). Proteins were dialysed in 1 x phosphate buffered saline (PBS) (pH 7.3) using Spectra/Por Float-A-Lyzer tubing with an 8000 to 10000 molecular weight cut off (MWCO) (Spectrum Laboratories, CA, USA), and LPS was removed using ProteoSpin endotoxin removal kit (Norgen Biotek, ON, Canada).

2.13.3 Protein quantification

Proteins were quantified using a nano-drop ND1000 spectrophotomer (Thermo scientific, USA) set on the A280 program. After the protein solution spectra were obtained, the concentration of the protein was calculated at an absorbance of 280 nm and the extinction coefficient calculated from the protein sequence as described previously (Gill and von Hippel, 1989).

Table 2.1 Protein purification buffers

Protocol	Buffer	Recipe	Final pH
Native conditions	Binding Buffer	50 mM NaH_2PO_4 300 mM NaCl 10 mM imidazole	8
Native conditions	Wash Buffer	50 mM NaH_2PO_4 300 mM NaCl 20 mM imidazole	8
Native conditions	Elution Buffer	50 mM NaH_2PO_4 300 mM NaCl 250 mM imidazole	8
Denaturing conditions	Binding Buffer	100 mM NaH_2PO_4 10 mM Tris·Cl 8 M urea	8
Denaturing conditions	Wash Buffer	100 mM NaH_2PO_4 10 mM Tris·Cl 8 M urea	6.3
Denaturing conditions	Elution Buffer	100 mM NaH_2PO_4 10 mM Tris·Cl 8 M urea	4.5

2.15 Western Immunoblot analysis

Proteins were separated by SDS-PAGE, as described in section 2.12 and transferred to nitrocellulose membranes (Amersham Hybond™ ECL™, GE Healthcare, Slough, UK) using Towbin transfer buffer (20 mM Tris, 154 mM Glycine and 20 % (w/v) Methanol (Fisher Scientific, UK)) and the Trans-Blot Turbo™ Blotting system (Bio-Rad, Hemel-Hempstead, UK), according to the manufacturer's specifications. Following transfer the membrane was incubated in 1 x PBS (pH 7.3) containing 8 % (w/v) powdered milk (Sigma-Aldrich, UK), at 4°C overnight. The membrane was incubated for 2 h with primary antibody at dilutions stated in each chapter then washed 3 times with washing buffer (1 x PBS (pH 7.3) containing 1 % (w/v) powdered milk and 0.05 % Tween 20 (v/v) (Sigma-Aldrich, UK). The membrane was then incubated with horse radish peroxidase-conjugated (HRP) secondary antibody for 1 h at the dilutions stated in each section. The membrane was washed and then incubated with ECL reagent for 5 min (ECL solution was composed of equal volumes of ECL 1 and ECL2, ECL1 contained 1 ml Luminol (Fluka, UK), 250 mM dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK), 0.44 ml p-Coumaric acid (Sigma-Aldrich, UK) 100 mM TrisHCl, with ddH_2O to a final volume of 100 ml. ECL solution 2 contained 64 µl 30 % H_2O_2 (Sigma-Aldrich, UK), 100 mM TrisHCl and ddH_2O to a final volume of 100 ml). Following incubation the membrane was visualized by exposing to Hybond ECL film (Amersham systems, Buckinghamshire, UK) for 20 s and processed in an X-ray developer (SRX-101A, Konic Minolta, Japan).

2.16 Isolation of human PMN and PBMC

50 ml tubes of venous blood was drawn and mixed with 6 ml of acid-citrate-dextran (ACD) (25 g D-glucose (Sigma-Aldrich, UK) and 20.5 g trisodium citrate (Sigma-Aldrich, UK) added to 1 L of ddH_2O). Blood was mixed 1:1 in 1 x PBS (Mg^{2+} Ca^{2+} free)

(Gibco, UK) and 25 ml was layered onto a dual layer Ficoll gradient (12 ml of a density 1.119 g/ml histopaque solution (Sigma-Aldrich, UK) in a 50 ml tube with 10 ml of density 1.077 g/ml ficoll-hypaque solution (GE healthcare, Buckinghamshire, UK) layered on top), prior to centrifugation for 20 min at 396 x g without brakes. Plasma was aspirated before the PBMC and PMN layers were recovered and each cell type was washed with RPMI 1640 (Gibco, UK) containing 0.05 % (v/v) human serum albumin (RPMI+HSA) (Sigma-Aldrich, UK), cells were pelleted by centrifugation at 400 x g for 10 min at 4°C. Contaminating red blood cells were removed from the PMN pellet by hypertonic lysis; the pellet was re-suspended in 9ml of ice cold ddH_2O and was incubated for 30 s. Isotonic conditions were restored by the addition of 1 ml of 10 x PBS (Gibco, UK) before washing in RPMI+HSA followed by centrigugation at 400 x g for 10 min at 4°C. Cells were counted using a TC20 automated cell counter (Biorad, UK) and re-suspended to a concentration of 1 x 10^7cells/ml.

2.17 T-cell proliferation assays

Human PBMC were adjusted to a concentration of 1x10^6 cells/ml in RPMI 1640 (Sigma Aldrich, UK) supplemented with 10% (v/v) foetal calf serum (Gibco, UK), 100 U/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml L-glutamine. (PSG) (Gibco, UK). Cells were cultured in 96 well low-bind round bottomed tissue culture plates (Nunc, Fisherbrand, UK) at 37°C in humidified air with 5 % CO_2. Proteins samples were tested in triplicate and added before incubation at the concentrations described in each chapter. Cells were cultured in medium only or with 5 μg/ml of Concanavalin A as negative and positive controls respectively. Proliferation of cells was determined using the incorporation of [^{3}H]-thymidine, by pulsing with 1 μCi/well of [^{3}H]-thymidine after a 72 h incubation and harvested after 18 h using a Tomtec Mach III M Harvester 96 (Hamden, USA) onto Wallac A filters (Perkin

Elmer, MA, USA). A Meltilex A wax scintillant strip (Perkin Elmer, MA, USA) was melted onto the filter pad and the [^{3}H]-thymidine incorporation into cellular DNA was determined by scintillation counting using a β-radiation counter (Wallac 1450 Microbeta PLUS, Perkin Elmer) and recorded as counts per minute (CPM).

Chapter 3

Structural and Expression Analysis of SEIX, SEIY and SEIZ

3.1 INTRODUCTION

The family of SAgs contains a number of characteristic motifs and structural features. To form the trimeric complex with the TCR and MHC class II each SAg contains a TCR binding site and MHC class II binding which cross link these receptors. SAgs contain one or both, of a low affinity MHC class II binding site and a high affinity site which uses zinc to coordinate binding (Fraser and Proft, 2008b). Recently a number of reports have suggested the existence of co-receptors on the surface of T-cells such as CD28 and CD40 which can contribute to T-cell activation (Brosnahan and Schlievert, 2011, Arad et al., 2011). All SAgs have also been shown to contain a motif termed the outside-in sequence, which is a dodecapeptide sequence at the base of the central α-helix involved in transcytosis of some SAgs across epithelial layers (Brosnahan and Schlievert, 2011).

A number of staphylococcal SAgs have also been demonstrated to induce emesis and are classified as staphylococcal enterotoxins (SEs). A common feature of SEs is a polypeptide cysteine loop framed by a di-sulphide bridge located in the N-terminal domain of the protein (Hu and Nakane, 2014). However the presence of this feature within the structure of a protein does not neccesarily correlate with emetic activity (Balaban and Rasooly, 2000, Hu and Nakane, 2014). Another property of SEs and staphylococcal SAgs is that they exhibit a high level of stability when exposed to varying temperature and pH conditions which enable the protein to transit through the human GI tract and retain activity (McCormick et al., 2001, Li et al., 2011).

An emerging theme in staphylococcal pathogenesis is that virulence determinants often exhibit more than one function (see section 1.5). There is a wide diversity in the SAgs encoded by *S. aureus* and it is possible that these proteins perform alternative functions independent of their T-cell mitogenicity during *S. aureus* infection. The SSl-proteins are a functionally diverse group of proteins that are structurally similar to SAgs but are non-

mitogenic (Fraser and Proft, 2008b). Accordingly it is feasible that SAgs may have some functional properties in common with SSl-proteins which could be reflected by conserved functional motifs.

In this study the structure of SElX, SElY and SElZ are analysed for the presence of motifs common to other SAgs. The proteins are also screened for motifs that could indicate functions other than superantigenicity. The regulation of expression of *selx* is also analysed to determine which gene regulators control *selx* expression and what signals may be involved in regulation during infection.

These aims will be achieved by:

- Building protein models and performing structural analysis of SElX, SElY and SElZ to determine the presence of features of SAgs and functional motifs found in other proteins
- Performing stability assessment and emetic studies to determine if any of these SAgs are enterotoxigenic
- Performing gene and protein expression analysis to investigate *selx* regulation

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and culture conditions

S. aureus strains (Table 3.1) were grown in tryptone soya broth (TSB) or brain heat infusion broth (BHI) (Oxoid, UK) shaken at 200rpm, or on tryptone soya agar (TSA) (Oxoid, UK) at 37°C for 16 h unless otherwise stated. *E. coli* strains were grown in Luria broth (LB) (Melford laboratories, UK) shaken at 200 rpm, or on LB-agar (Melford laboratories, UK) at 37°C for 16 h unless otherwise stated. Media was supplemented, where appropriate, with antibiotics. For selection transformed *E. coli* 100 µg/ml ampicillin was used. For the selection of *S. aureus* muntants containing insertion sequences tetracycline (5 µg/ml) or erythromycin (10 µg/ml) were added to the media as required. Strains were stored in appropriate liquid culture media containing 40% (v/v) glycerol (Sigma-Aldrich, UK) in cryovials (Nunc, Thermo Scientific, UK) at -80°C.

3.2.2 Cloning and purification of recombinant protein

Gene templates were amplified by PCR using the proof reading polymerase Pfu fusion II polymerase (Agilent technologies, UK) with primers stated in Table 3.2. Genomic DNA from *S. aureus* strains MSA2020 and MSA1695 were used as templates for *sely* and *selz* respectively. The *sea* gene was amplified from wild type *S. aureus* Newman and *tst* was amplified from the ED84 isolate (Table 3.1). The PCR product was cloned into the pSC-B plasmid using Strataclone blunt cloning kit (Aglient, technologies, UK) as described in the manufacturer's protocol. Following cloning the pSC-B plasmids were isolated using the QIAprep minikit (Qiagen, Manchester, UK) and digested with appropriate restriction enzymes. The inserts were gel-purified after agarose gel electrophoresis using the Qiaquick gel purification kit (Qiagen, Manchester, UK) and ligated into the desired plasmid with T4 DNA ligase (New England Biolabs, Herts, UK) for 16 h at 16°C. The ligations were dialysed

on 0.025 µm filter discs (Millipore, Watford, UK) and transformed into *E. coli* strains DH5α or XL-1 blue. For recombinant protein expression the plasmids were and transformed into *E. coli* BL21 DE3 by electroporation according to the general methods section 2.11.

Plasmid pET15b and pQE30 constructs containing SAg genes were used for the expression of recombinant proteins (Table 3.3). Proteins expression was induced as outlined in section 2.13.1. All recombinant proteins were purified using the native purification protocol outlined in section 2.13.2 except SElX and SSl5. SElX was purified using the denaturing protocol outlined in section 2.13.2 followed by a second purification step using IEC also described in section 2.13.2. SSl5 was purified with a hybrid purification protocol as follows; induced cells were lysed and bound to the affinity column with denaturing protocol buffers (Table 2.1) using the protocol described in section 2.13.2 and then washed with the denaturing wash buffer as described in 2.13.2. Protein is then refolded on the column by adding native protocol wash buffer over a gradient (30 min to 100% at a flow rate of 1 ml/min). The protein was eluted with native protocol elution buffer (Table 2.1).

3.2.3 Phylogenetic and amino acid sequence analysis

The sequences of SAgs and SSl-protein genes were obtained from GenBank (Table 3.4). The Nucleotide sequences were aligned using BLOSUM (Blocks substitution matrix) 62 in MEGA 5.2 software. The sequence alignment was used to construct maximum-likelihood phylogeny. Predicted structural models were built using the I-TASSER server (Roy et al., 2011) and crystal structures were downloaded from the RCSB PDB databank. Structural models were analysed in PyMol (DeLano, 2002).

Table 3.1 Bacterial strains used in this study.

Strain	Description	Source/ Reference
S. aureus		
Newman	Wild type (agr+)	(Duthie and Lorenz, 1952)
VJT 7.17	Newman *agr*::*tet*	(Benson et al., 2011)
VJT 9.98	Newman *rot*::*Tn917*	(Benson et al., 2011)
VJT 10.03	Newman *agr*::*tet rot*::*Tn917*	(Benson et al., 2011)
VJT 12.22	Newman *saeS*::*bursa*	(Benson et al., 2011)
VJT 17.25	Newman *agr*::*tet* *saeS*::*bursa*	(Benson et al., 2011)
USA300 LAC	WT Erythromycin sensitive	(Benson et al., 2011)
AH1292	USA300 LAC *agr*::*tet*	(Benson et al., 2011)
VJT 16.37	USA300 LAC *agr*::*tet* *rot*::*Tn917*	(Benson et al., 2011)
VJT 28.25	USA300 LAC *saeS*::*bursa*	(Benson et al., 2012)
MSA2020	Human SSS clinical isolate, France	(Musser and Selander, 1990)
MSA1695	Human SSS clinical isolate, Japan	(Musser and Selander, 1990)
ED84	Chronic cystic fibrosis infection	(McAdam et al., 2011)
E. coli		
DH5α	Cloning strain	Invitrogen, Paisley, UK
BL21 DE3	Expression strain	Invitrogen, Paisley, UK
XL-1 blue	Cloning and expression strain	Agilent Technologies, UK
Strataclone SoloPack™	*lacZΔM15* mutation, *endA*, *recA* deficient	Agilent Technologies, UK

Table 3.2 Oligonucleotide primers used in this study.

Primer	Forward Sequence (5'-3')[a]	Reverse sequence (5'-3')[a]	Restriction Enzymes
Cloning			
*sea*pET	CCCCATATGAGCGAAAAAAGCGAAG	CCCGGATCCTTAGCTGGTATACAGATAAAT	*Nde*I and *Bam*HI
*tst*pET	CCCCATATGTCTACAAACGATAATATAAAGG	CCCCTCGAGTTAATTAATTTCTGCTTC	*Nde*I and *Xho*I
*selyhu*pET	GCGCCATATGAAAACAACTGGATTGATTA	GCGCGGATCCCTATTTCATATAAATATC	*Nde*I and *Bam*HI
*selzhu*pQE	TAGCGGATCCGAGACACAAAATGATCCAAA	GCGCCTGCAGCTACTTTTTAGTTAAGT	*Bam*HI and *Pst*I
qRT-PCR			
16rRNA (Wilson *et al*, 2011)	TATGGAGGAACACCAGTGGCGAAG	TCATCGTTTACGGCGTGGACTAAC	
selxq (Wilson *et al*, 2011)	AGCAGACGCGTCAACACACAAA	GGTCTCTCTGAATAAACCCAATTCC	

[a] Restriction sites incorporated are underlined

Table 3.3 Plasmids used in this study.

Plasmid	Description	Source/ Reference
pET15b	Overexpression plasmid encoding an N-terminal 6 x HIS-tag	Invitrogen, Paisley, UK
pET15b::*selx2*	*selx2* cloned into pET15b	(Wilson et al., 2011)
pET15b::*sea*	*sea* cloned into pET15b	This Study
pRSETB::*ssl5*	*ssl5* cloned into pRSETB	(Bestebroer et al., 2007)
pET15b::*ssl7*	*ssl7* cloned into pET15b	(Wilson et al., 2011)
pET15b::$sely_{hu}$	$sely_{hu}$ cloned into pET15b	This Study
pet15b::*tst*	*tst* cloned into pET15b	This Study
pQE30-xa	Overexpression plasmid encoding an N' terminal 6 x HIS-tag	Qiagen, Manchester, UK
pQE30-xa::$selz_{hu}$	$selz_{hu}$ cloned into pQE30-xa	This Study

3.2.4 Circular dichroism analysis

Far UV circular dichroism (CD) spectra of protein samples was recorded in 0.1 x PBS (pH 7.3) on the Jasco J-710 (Japan Spectroscopic Co. Ltd, Japan). Spectra of the proteins, at a concentration of 0.4 mg/ml to 1 mg/ml, were recorded between 190 nm to 250 nm using a cuvette with a path length of 0.05 mm. For analysis the precise molar concentration was determined for each protein and used in the Jasco software to normalise each protein spectra before the data was analysed in further detail.

3.2.5 Thermal shift analysis

To monitor protein unfolding, the fluorescent dye Sypro orange (Life technologies, UK) was used. The unfolding process exposes the hydrophobic region of proteins and results in a large increase in fluorescence that can be used to monitor the protein-unfolding transition. The thermal shift assay was conducted in a Lightcycler 480 (Roche, West Sussex, UK) using the melt curve function determined from 25°C to 95°C. Solutions of 5 μM recombinant staphylococcal protein were mixed with Sypro orange dye diluted 1/125 of the stock dye. The plate was heated from 25°C to 95°C with a heating rate of 0.1°C/sec. The fluorescence intensity was measured with excitation/emission: 465/510 nm.

3.2.6 Western blot analysis

Supernatants from stationary phase (16 h) cultures of *S. aureus* strains grown in BHI broth were concentrated with Amicon Ultra-15 Centrifugal Filter units (10000 MWCO) (Millipore, Watford, UK). Concentrated secreted proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes using the Trans-Blot Turbo™ Blotting system (Bio-Rad, Hemel

Hempstead, UK), according to the manufacturer's specifications. The membrane was incubated in 1 x PBS (pH 7.3) containing 8 % (w/v) powdered milk (Sigma-Aldrich, UK), at 4°C overnight. The membrane was incubated with primary antibody (Anti-SElX chicken IgY), at a concentration of 1:1000 for 2 h. Anti-SElX IgY was obtained from immunised hen's eggs using the Eggspress™ IgY purification kit (Gallus immunotech, NC, USA) according to the manufacturer's instructions. Following primary antibody incubation the membrane was washed 3 times with washing buffer, 1 x PBS (pH 7.3) containing 1 % (w/v) powdered milk and 0.05 % (v/v) Tween 20 (Sigma-Aldrich, UK). The membrane was then incubated with horse radish peroxidase-conjugated (HRP) secondary antibodies (goat anti-chicken-IgY IgG (Source Bioscience, Nottingham, UK)), diluted 1:10000 in washing buffer, for 1 h, followed by a further 3 washes and then incubated with ECL reagent for 5 min. The blot was then exposed on Hybond ECL film (Amersham, Systems, Buckinghamshire, UK) for 20 s and developed using an X-ray developer (SRX-101A, Konic Minolta, Japan).

Table 3.4 *S. aureus* whole genome sequences, plasmids and gene sequences examined from publically available sources.

Strain/ Plasmid/Gene	NCBI Reference Number	Geographical Origin	Sequence Type (ST)	Reference
Newman strain	NC_009641.1	UK, 1950	8	(Duthie and Lorenz, 1952)
RF122 strain	NC_007622	Ireland, 1993	151	(Herron-Olson et al., 2007b)
ED98 strain	NC_013450.1	UK, 1996	5	(Lowder et al., 2009b)
COL strain	NC_002951.2	UK	250	(Gill et al., 2005b)
FPR3757 strain	NC_007793.1	USA, 2003	8	(Diep et al., 2006)
Mu50 strain	NC_002758.2	Japan, 1997	5	(Kuroda et al., 2001)
MW2 strain	NC_003923.1	USA, 1998	1	(Baba et al., 2002)
pSK67-M1 Plasmid	HF937104.1	Denmark, 2003		(Larner-Svensson et al., 2013)
pF5 plasmid	NG_036541.1	Japan, 2008		(Ono et al., 2008a)
selv gene	EF030427.1			(Thomas et al., 2006)
seu gene	AY205305.1			(Letertre et al., 2003)
see gene	M21319.1			(Couch et al., 1988)
selw gene	EF030428.1			(Thomas et al., 2006)

3.2.7 qRT-PCR analysis

3.2.7.1 RNA extraction from *S. aureus*

Total RNA was extracted from post-exponential phase (OD_{600} = 4.0 to 7.0) *S. aureus* cultures using the RNeasy miniprep plus kit (Qiagen, Manchester, UK) according to the manufacturer's specification except for a modified lysis step, that involved the re-suspension of the washed cells in Tris-EDTA (TE) buffer with 100 µg/ml lysostaphin (Ambi Products, USA) and incubation at 37°C for 30 min. RTL buffer from the RNeasy miniprep plus kit (Qiagen, Manchester, UK) was added to the cells and they were disrupted using the FastPrep® FP 120 machine (MP Biomedicals, UK) in tubes containing lysis matrix B (MP Biomedicals, UK). Following disruption tubes were centrifuged at 13,000 x g for 1 min to remove the matrix and then RNA was isolated from the supernatant using the RNeasy plus kit (Qiagen, Manchester, UK). RNA was treated with the Turbo-DNA-Free kit (Ambion, Life Technologies, UK) as described in the manufacturer's protocol. 1% (w/v) agarose gel electrophoresis of RNA samples was carried out to verify the integrity of the RNA. RNA was quantified and purity was determined using a nano-drop ND1000 spectrophotometer (Thermo scientific, UK).

3.2.7.2 Quantitative reverse transcriptase-PCR

qRT-PCR reactions were performed using the power SYBR green® RNA-to-Ct™ 1-step kit (Life technologies, UK) with a lightcycler 480 (Roche, West Sussex, UK). qRT-PCR primer sequences and their concentrations were obtained from previously published work (Table 3.2). Each primer set is designed to amplify a DNA fragment between 150 bp and 200 bp, and 50 ng of isolated RNA was used as a template in a 25µl reaction. RNA samples were processed in triplicate with a no template control (NTC), no RT controls and gDNA standards. The thermocycling conditions were 48°C for 30 min for 1 cycle, 10 min at 95°C for 1 cycle,

followed by 45 cycles of 20 s at 95°C, 20 s at 55°C and 20 s at 72°C with fluorescence measured at the end of each annealing step.

3.2.8 House musk shrew model of emesis

To determine the emetic capacity of SElX and SElZ, recombinant proteins were tested in a house musk shrew model of emesis at the Hirosaki University as previously described (Hu et al., 2003). Briefly, emetic assays were performed with healthy adult (2 to 10 m-old) house musk shrews (*Suncus murinus*) weighing 50 g to 70 g (male) and 40 g to 50 g (female). Each animal was placed in a separate cage, fed on commercial feed formulated for *S. murinus* (Nihon Clea, Tokyo, Japan), and provided water *ad libitum*. Purified proteins were diluted in 0.01 M PBS (pH 7.2), and 200 µl volumes of proteins at an appropriate dilution were administered intraperitoneally to the house musk shrews. The animals were observed for emesis for 3 h after the intraperitoneal administration of proteins. The number and times of vomiting, the time to the first vomiting episode, and any behavioural changes were recorded.

3.3 RESULTS

3.3.1 Distribution and genetic relation of SElY and SElZ.

The staphylococcal SAgs are a genetically diverse group with sequence homology ranging from 86 % (SEA versus SEE) to 26 % (SEA versus TSST-1) identity. Despite this the staphylococcal SAgs form distinct groups when analysed phylogenetically (Spaulding et al., 2013). Alignments of all characterised SAgs and the recently identified SAgs *sely* and *selz* were analysed by constructing a maximum likelihood tree (Fig 3.1). This phylogeny demonstrates that *selx* segregates outside the major groups of SAgs with an outlier group more closely related to the SSl-proteins as previously reported by Wilson et al. (2011). This outlier group includes *tst*, *set* and *sely*, and is more closely related to the SSl-proteins than to other SAgs. SSl-proteins classically exhibit diverse immune modulatory functions and the possibility that *selx* and *sely* exhibit these functions warrants further investigation.

Further sequence analysis was performed on *sely* using an in-house database of 813 sequenced *S. aureus* genomes. The gene sequence of *sely* was compared against whole genome sequences available using BLAST (basic local alignment search tool) This analysis demonstrated 91 out of 813 *S. aureus* genomes (11.1%) carried the *sely* gene. The dataset also identified 20 different allelic variants of *sely* and 39 positions varied within the protein sequences of the different alleles (Appendix 1.1). The most diverse allelic variants were found to share 91.442 % identity (SElY1 versus SElY19) (Appendix 1.1). The in house data base contains genome sequences from *S. aureus* isolated from a range of species and the *sely* gene was found in strains associated with many of these different host species including humans, cattle and rabbits.

The novel SAg *selz* is most closely related to *seg* (41 % identity) and this SAg falls within the group II SAgs (Fig 3.1). This group exhibits 2 zinc binding sites and many members of this group have been shown to be emetic. The relatedness of *selz* to SAgs like *seg, ser* and

sec suggest that *sely* is a more classical SAg compared to *selx* and *sely*. Using the in-house *S. aureus* genome database the *selz* gene was identified in 54 out of 813 *S. aureus* genomes (6.7%) using BLAST. Sequence analysis of the SElZ protein demonstrated there were 11 allelic variants of this protein and the gene appears to be highly conserved (Appendix 1.2) as the most diverse variants were found to share 94.981 % identity (SElZ3 versus SElZ10). There are 19 positions within the SElZ protein sequence that exhibit variation and 7 of these occur in the signal sequence region of the protein (Appendix 1.2), if this region of the protein region is excluded then sequence identify between SElZ3 and SElZ10 increases to 97.242 %.

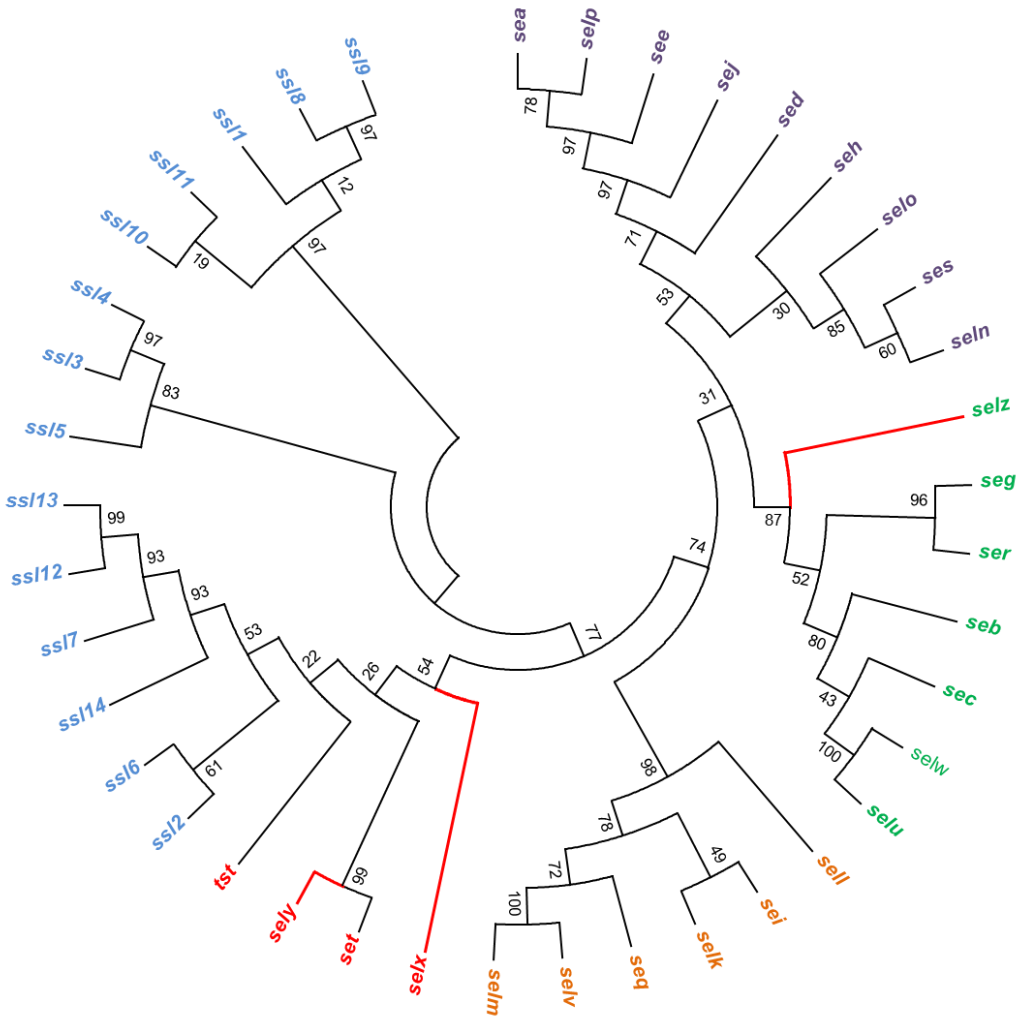

Figure 3.1 Phylogenetic analysis of *selx*, *sely*, and *selz*. The genetic relatedness of staphylococcal SAgs and SAg-like proteins was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-14825.7934) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 40 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 402 positions in the final dataset. Analyses were conducted in MEGA5 (Tamura et al., 2011). Genes are coloured according to the group classification described previously (Spaulding et al., 2013). Blue genes denote the SSl proteins, red show the group I SAgs, green genes denote the group II SAgs, group III SAgs are coloured purple and group V SAgs are coloured orange. The branches for *selx*, *sely* and *selz* have been coloured red to highlight their position in the tree.

3.3.2 SElX, SElY and SElZ exhibit features associated with other SAgs.

The crystal structure of SElX, SElY and SElZ have yet to be determined, so structure predictions of the proteins were made using the I-Tasser server (Roy et al., 2011). Protein motifs in the sequences of SElX, SElY and SElZ were determined by aligning the amino acid sequences with that of characterised SAgs. Analysis of the protein models was combined with protein alignments to look for analogous residues and motifs from other SAgs that may contribute to the function of the protein (Fig 3.2 and Fig 3.3). The models of SElX, SElY and SElZ all exhibit the two domain structure of other SAgs as typified by the crystal structure of TSST-1 (Fig 3.2 and Fig 3.3). Sequence alignments demonstrate that all three SAgs analysed share residues that could interact with the T-cell receptor (Fig 3.2). Each of the SAgs encode a variant of the outside-in signalling motif which has been implicated in co-receptor signalling in T-cell activation (Fig 3.2, Fig 3.3 and Fig 3.4) (Arad et al., 2011, Brosnahan and Schlievert, 2011, Spaulding et al., 2013). This motif is found at the base of the conserved central alpha helix in SElX, SELY and SElZ, similar to all other SAgs examined to date (Fig 3.4).

The position of predicted MHC class II binding residues is more diverse in the three SAgs analysed. SElX is predicted to encode only the low affinity MHC class II site found within the N-terminal OB domain. This domain is predicted to be highly truncated in SElX suggesting that a novel MHC class II interaction may occur. SElZ is predicted by the I-TASSER software to interact with zinc, indicative of a SAg that binds to the MHC class II via the high affinity site (Abrahmsen et al., 1995, Hudson et al., 1995). The location of the zinc binding site is predicted to be in the C-terminal grasp domain as observed in other zinc binding SAgs such as SEA and SEC. A second zinc binding site was also predicted in the OB fold domain of SElZ. SAgs with two zinc-binding sites such as SEC have been shown to form dimers that allow for cross linking of MHC class II complexes on the surface of APC cells (Chi et al., 2002). SElZ exhibits a loop structure in the N-terminal domain which is framed by two parallel cysteine residues (Fig 3.3) a characteristic feature of many emetic SAgs (Balaban and Rasooly, 2000).

This suggests SElZ may behave as an enterotoxin. SElY is predicted to encode the low affinity MHC class II binding site in the OB domain. This protein is predicted to exhibit a structure very similar to TSST-1 (Fig 3.2 and 3.3)

3.3.3 SElX contains a conserved sialic acid binding motif.

If SAgs perform alternative functions during *S. aureus* infection then it is possible they contain protein motifs associated with these functions. Sequence alignments of SElX with previously characterised SSl-proteins revealed a conserved sialic acid binding motif (Fig 3.5) (Baker et al., 2007, Chung et al., 2007, Hermans et al., 2012). The motif in SElX has a 54% amino acid sequence identity with the same motif found in SSl5. Of note, this motif is commonly found in staphylococcal proteins that interact with neutrophils (Hermans et al., 2012). Within the motif are 4 amino acid residues that have been demonstrated to interact with sialic-Lewis X (sLEX) (Hermans et al., 2012), and 3 of these residues (E4, K7, and Q10) are conserved in all 5 proteins identified to date that contain this motif (Fig 3.5). The fourth residue shown to interact with sLEX in crystallography studies varies among the SSl-proteins and allelic variants of SElX suggesting that this residue may confer some level of specificity to the protein but is not essential for function (Fig 3.5).

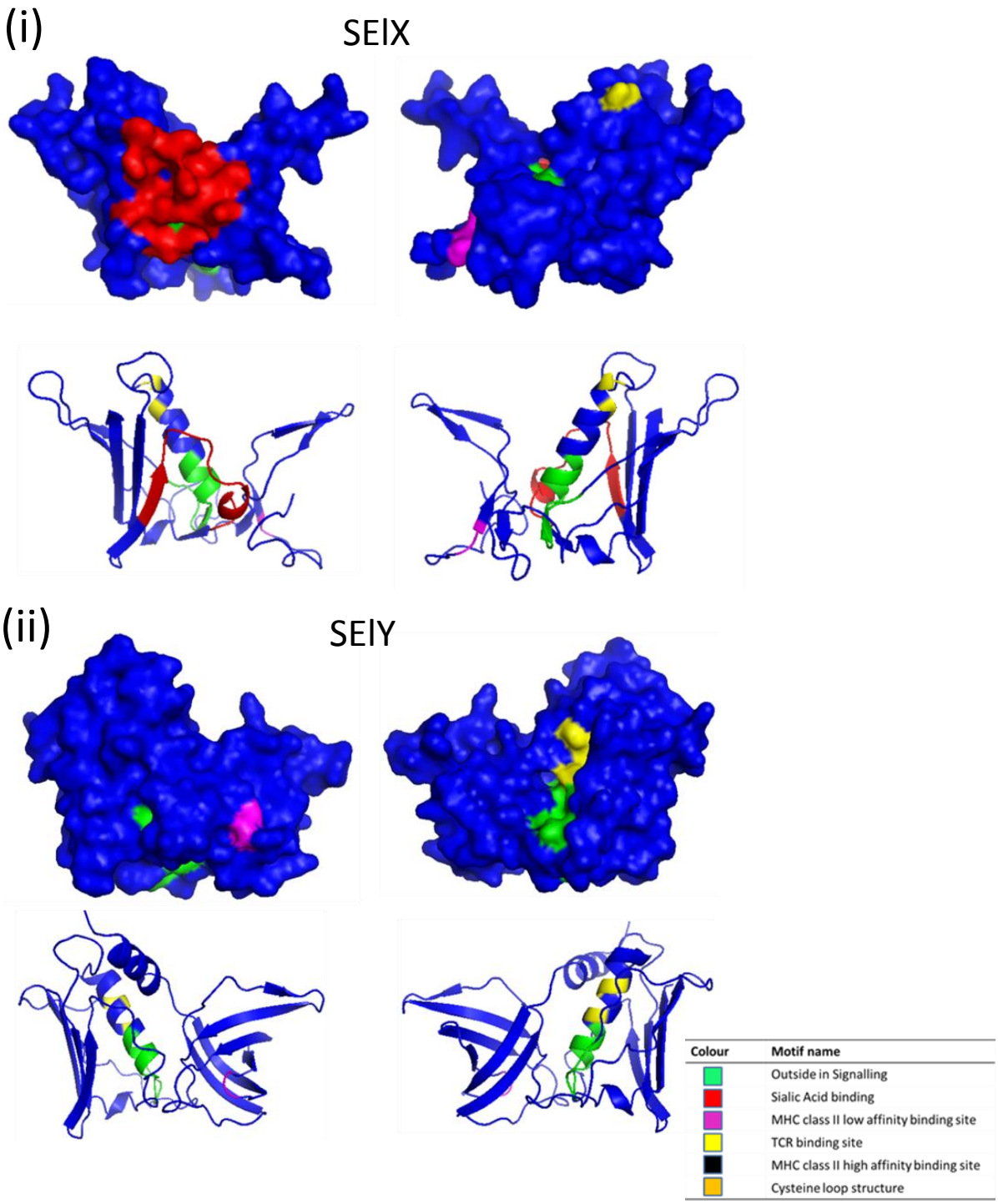

Figure 3.2 SEIX and SEIY share a number of common features with other characterised SAgs.(i) Model structure of SEIX. (ii) Model structure of SEIY. Front and rear views (right and left respectively) are shown in surface and cartoon forms (top and bottom respectively). Key features of the proteins are coloured and described in the key. Alignments of protein sequences showing the position of the residues within the proteins sequences are shown in appendix 1.3-1.5

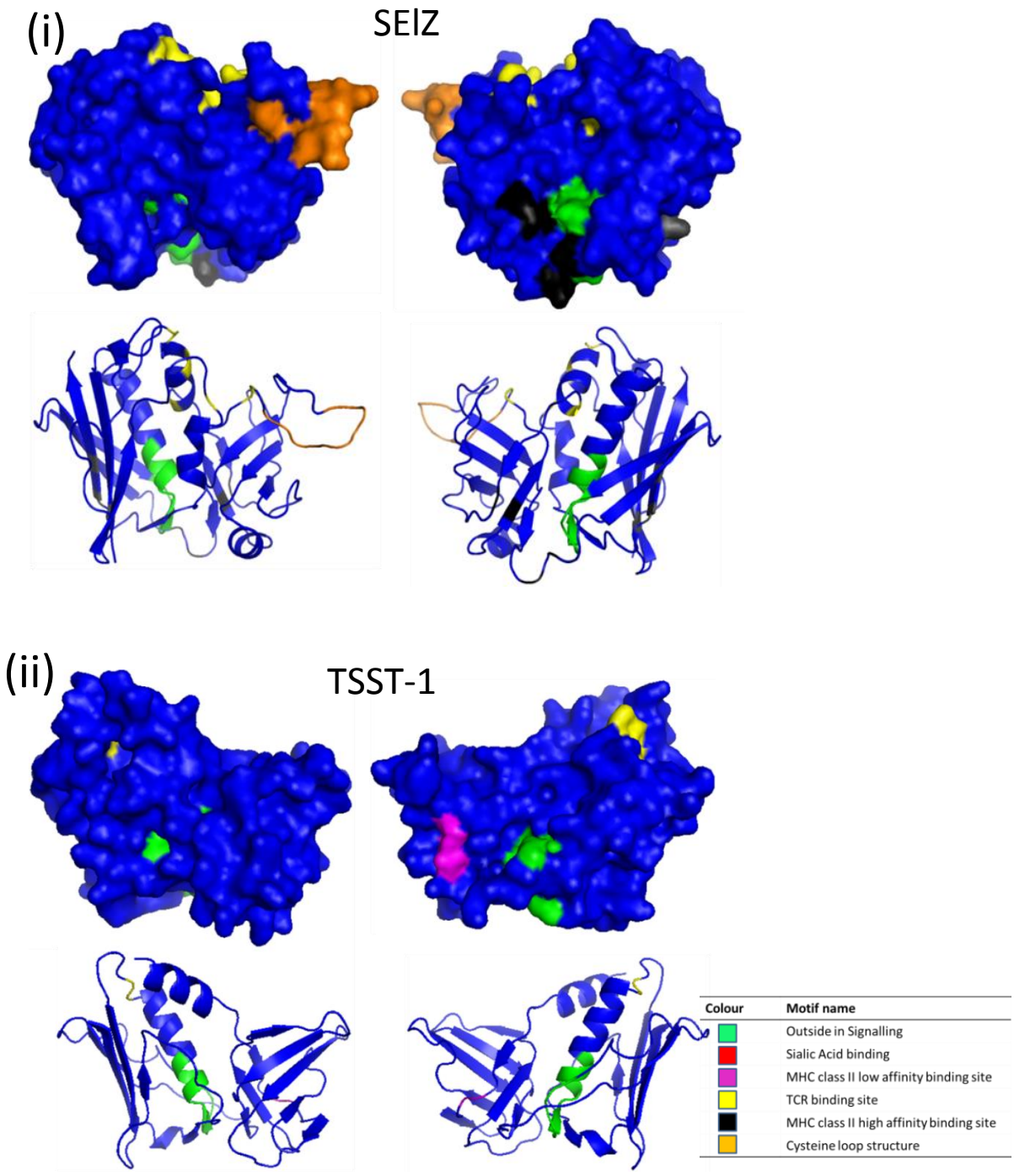

Figure 3.3 SElZ shares a number of common features with other characterised SAgs. (i) Model structure of the SAg SElZ. (ii) The crystal structure of TSST-1. Front and rear views (right and left respectively) are shown in surface and cartoon forms (top and bottom respectively). Key features of the proteins are coloured and described in the key. Alignments of protein sequences showing the position of the residues within the proteins sequences are shown in appendix 1.3-1.5

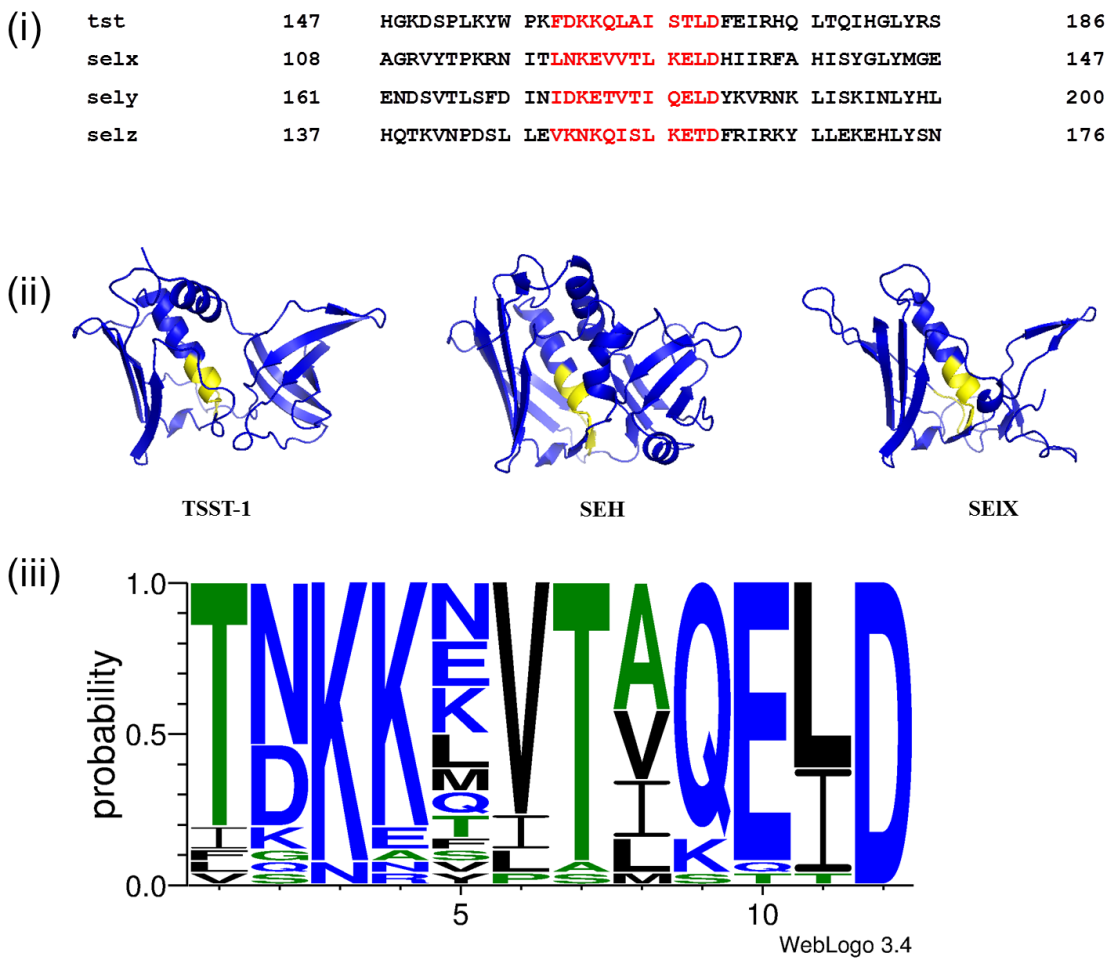

Figure 3.4 SElX, SElY and SElZ all encode the outside-in signalling sequence. (i) Amino acid sequence alignments of the outside-in signalling sequence (coloured red) of TSST-1, SElX, SElY and SElZ showings the relative position of this motif in the sequence. (ii) Ribbon diagrams of three staphylococcal SAgs demonstrate the outside-in motif (coloured yellow) is consistently located at the base of the central α-helix. (iii) Amino acid residues within the outside-in signalling sequence from all characterised SAgs including SElX, SElY and SElZ were used to show the conservation of specific residues among various proteins. Residues more likely to be at each position appear larger than their less common counterparts. Image generated using the free weblogo 3.4 program located at http://weblogo.threeplusone.com/. Hydrophobicity colour scheme: blue is hydrophobic, green is hydrophilic and black is neutral.

(i)

```
Conserved                    ** *   * **  *     *    *
SSl3        322     NGGKYTFELH KKLQEHRMAD VIDGTNIDNI EVNIK      356
SSl4        274     NGGKYTFELH KKLQENRMAD VIDGTNIDNI EVNIK      308
SSl5        200     DGGYYTFELN KKLQTNRMSD VIDGRNIEKI EANIR      234
SSl6        195     DDKFYTFELT KKLQPHRMGD TIDGTKIKEI NVELE      231
SSl11       192     DGGFYTFELN KKLQTHRMGD VIDGRNIEKI EVNL*      226
SElX        160     DGGKYTLESH KELQKDRENV KINTADIKNV TFKLV      194
```

(ii)

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Conserved                 ********* ***** ****   ** ***** ** **
SElX1       160     DGGKYTLESH KELQKDRENV KINTADIKNV TFKLV      194
SElX2       160     DGGKYTLESH KELQKDRENV KINTADIKNV TFKLV      194
SElX3       160     NGGKYTLESH KELQKNRENV EINTDDIKNV TFELV      194
SElX4       160     DGGKYTLESH KELQKNRENV KINTADVKNV TFELV      194
SElX5       160     DGGKYTLESH KELQKDRENV KINTADIKNV TFKLV      194
SElX6       160     DGGKYTLESH KELQKDRENV KINTADIKNV TFDLV      194
SElX7       160     NGGKYTLESH KELQKDRENV KINTDDIKNV TFELV      194
SElX8       160     NGGKYTLESH KELQKNRENV EINTDDIKNV TFELV      194
SElX9       160     NGGKYTLESH KELQKDRENV KINTDDIKNV TFELV      194
SElX10      160     NGGKYTLESH KELQKDRENV KIKTDDIKNV TFELV      194
SElX11      160     NGGKYTLESH KELQKDRENV KINTDDIKNV TFELV      194
SElX12      160     DGGKYTLESH KELQKDRENV KINTDDIKNV TFELV      194
SElX14      160     DGGKYTLESH KELQKDRENV EINTADIKNV TFELV      194
SElXbov     160     NGGKYTLESH KELQKNRENV EINTDDIKNV TFELV      194
SElXbov2    160     NGGKYTLESH KELQKDRENV KINTDDIKNV TFELV      194
SElXov      160     NGGKYTLESH KELQKDRENV KNNTDDIKNV TFELV      194
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(iii)

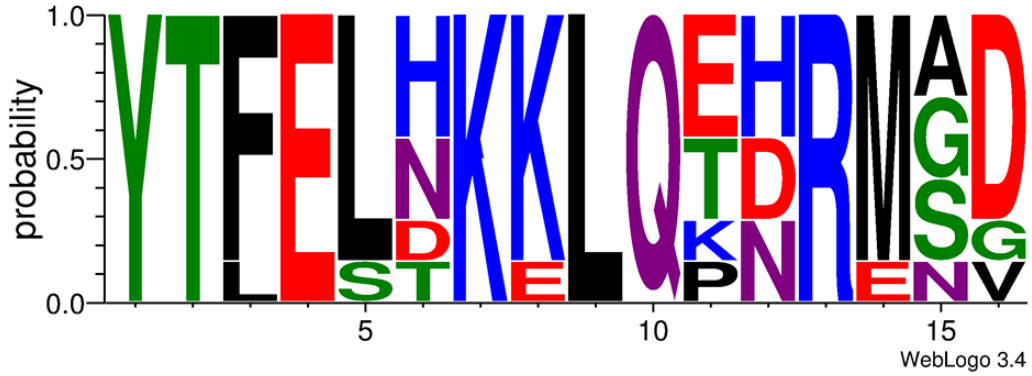


Figure 3.5 SElX protein sequence encodes a conserved sialic acid binding motif. (i) Amino acid sequences of characterised SSl-proteins and SElX reveal a conserved glycan binding motif (coloured red). (ii) The sialic acid binding motif is conserved in all 17 alleles of SElX. (iii) Amino acid residues within the sialic acid binding region of 7 staphylococcal neutrophil binding proteins (SSl2, SSl3, SSl4 SSl5, SSl6, SSl11 and SElX) were used to show the conservation of specific residues among various proteins. Residues more likely to be at each position appear larger than their less common counterparts. Image generated using the free weblogo 3.4 program located at http://weblogo.threeplusone.com/. Chemical property colour scheme: green is polar, blue is basic, red is acidic, purple is neutral and black is hydrophobic.

3.3.4 SElX, SElY and SElZ circular dichroism analysis suggest novel features.

The models of SElY and SElZ suggest they may have a structure similar to that of previously characterised SAgs. In contrast SElX is predicted to contain a truncated N-terminal domain which suggests a novel mechanism of MHC class II interaction. Recombinant proteins were prepared and tested for the ability to induce non-specific human T-cell proliferation by thymidine incorporation (Fig 3.6). The five SAgs; SElX, SElY, SElZ, SEA and TSST-1 were all demonstrated to be able to induce non-specific T-cell mitogenicity (Fig 3.6). All the SAgs except SElX proved to be highly potent to the point that they were capable of inducing T-cell proliferation at concentrations as low as 100 pg/ml (Fig 3.6). SElX induced T-cell proliferation at concentrations higher than 10 ng/ml, indicative of lower potency compared to other SAgs. This is the first time that SElZ has been demonstrated to be mitogenic for human T-lymphocytes. The 5 SAgs were then analysed using circular dichroism (CD) to examine secondary structure content (Fig 3.7). By including structurally characterised SAgs in this analysis the CD spectra may provide an insight into the structures of SElX, SElY and SElZ.

The secondary structure content of the 5 SAgs and SSl7 was determined by CD. All 6 proteins tested exhibited varying CD spectra which are indicative of variable structures (Fig 3.7). The data acquired from this analysis was found to be unreliable below 200nm due to the buffer conditions and equipment used, the lamp used was a standard Xe lamp and buffers were not degassed prior to use which can both contribute to noise below 200 nm (Kelly et al., 2005). Due to these limitations, accurate estimation of secondary protein structure was not possible. However these data can still be compared to spectra from proteins that exhibit 100 % of each secondary structure (Greenfield, 2006). Overall SSl7 gave a relatively weak spectrum and it is difficult to determine which secondary structure predominates but it appears to be distinct from the other proteins analysed (Fig 3.7). The CD spectra of TSST-1 indicated a mixture of helices, beta sheets and a strong signal for random coil (signal observed between 200 and 210nm), which is demonstrated in the crystal structure of TSST-1 (Fig 3.3 and Fig 3.7). This

demonstrates that the CD spectrum gives an accurate reflection of secondary structure in these recombinant proteins. For SElX the predicted structure is predominated by random coils and for SElY the structure is predicted to be more mixed between different secondary structures (Fig 3.2). The models of SElX and SElY were built using a variety of Sag and SSls as a template. Given that neither of these proteins exhibit a CD spectra remotely close to that seen for TSST-1 or SSl7 it suggests that these proteins exhibit a different structure than that predicted (Fig 3.7). SElX and SElY CD spectra suggest proteins that exhibit a structure predominated by α-helices which does not reflect the models (Fig 3.7). SElZ and SEA CD spectra both indicate proteins predominated by β-sheets which is similar to the secondary structure predicted in the model for SElZ and shown by the crystal structure of SEA (Fig 3.7 and Fig 3.3). These proteins display a diverse range of secondary structure and the analysis has demonstrated that the predicted models of SElX and SElZ may be inaccurate.

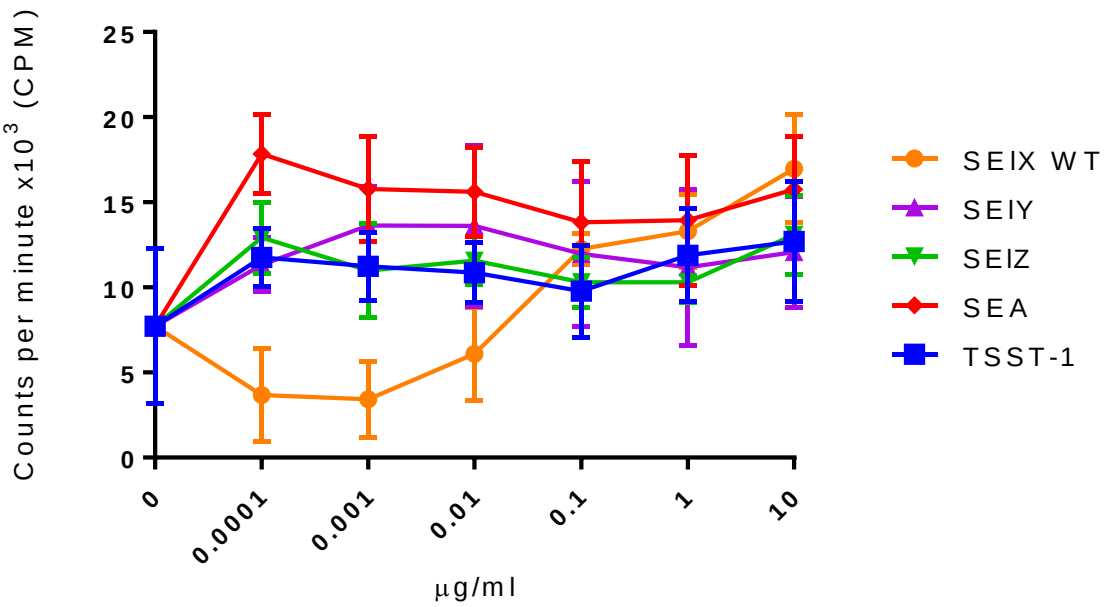


Figure 3.6 Recombinant staphylococcal superantigen proteins tested induce T-cell proliferation. Human PBMC proliferation assay after 72 h exposure to recombinant SAgs, as indicated by the incorporation of [^{3}H] thymidine. Results shown are the means of triplicate measurement from 3 human donors ± standard deviation of the mean.

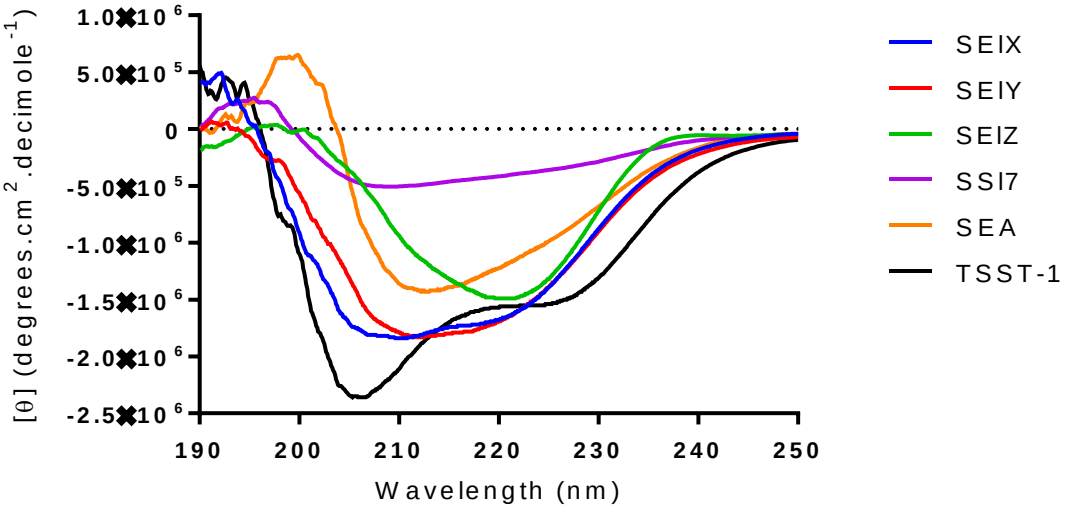


Figure 3.7 Staphylococcal SAgs exhibit distinct CD spectra. Recombinant staphylococcal proteins were used to generate far UV CD spectra of secondary structure. Each spectrum was normalised to the precise molar concentration analysed and compared to spectra from the other recombinant proteins.

3.3.5 SElX, SElY and SElZ exhibit a high level of stability.

Staphylococcal SAgs have classically demonstrated a very high level of stability in response to variations in pH and temperature (Li et al., 2011). This property of the staphylococcal enterotoxins facilitates survival in the human GI tract prior to the induction of emesis (Hu and Nakane, 2014). In the current study, to determine if the novel SAgs also exhibit this property, staphylococcal proteins (SElX, SElY, SElZ, SEA, TSST-1 and SSl5) were dialysed in PBS buffer of varying pH (4 to 9). The thermostability profile of each protein under each condition was determined using thermal shift analysis (Fig 3.8), demonstrating distinct spectra under different pH conditions.

The most stable proteins are SElY and TSST-1, as measured by a lack of a phase shift in fluorescence up to 50°C. The most unstable protein is SSL5 as this protein exhibited spectra indicative of complete degradation above pH 5. The other three proteins (SElX, SElZ and SEA) all gave more intermediate profiles. A feature of the SEA and SElZ profiles was biphasic protein degradation across the pH spectrum implying that there are structures within the protein that are more thermostable than others (Fig 3.8). SElX and TSST-1 also exhibit biphasic profiles but these only occur under certain pH conditions (Fig 3.8).

The T_m of each protein under each condition was calculated (this is defined as 50% of maximum unfolding) and in the event of biphasic degradation the T_m was calculated for the first phase of the degradation only. These data were plotted against pH (Fig 3.9) indicating that the melting temperature varies only slightly over the pH range tested (Fig 3.9). SEA was the only protein to demonstrate any relationship between stability and pH with a slight increase in T_m at the higher pH buffers tested (Fig 3.9) and the other proteins remain stable over a wide pH range. SElY and TSST-1 are the most stable proteins tested with a mean T_m in excess of 50°C. SElX and SElZ demonstrate a high level of resistance to changing pH conditions as the T_m of these proteins exhibit little variation from pH 4 to pH 9.

Taken together the SAgs SElX, SElY and SElZ all exhibit a T_m in excess of 40°C and demonstrate resistance to variations in pH, conditions that a potentially emetic protein may be exposed to during GI tract transit.

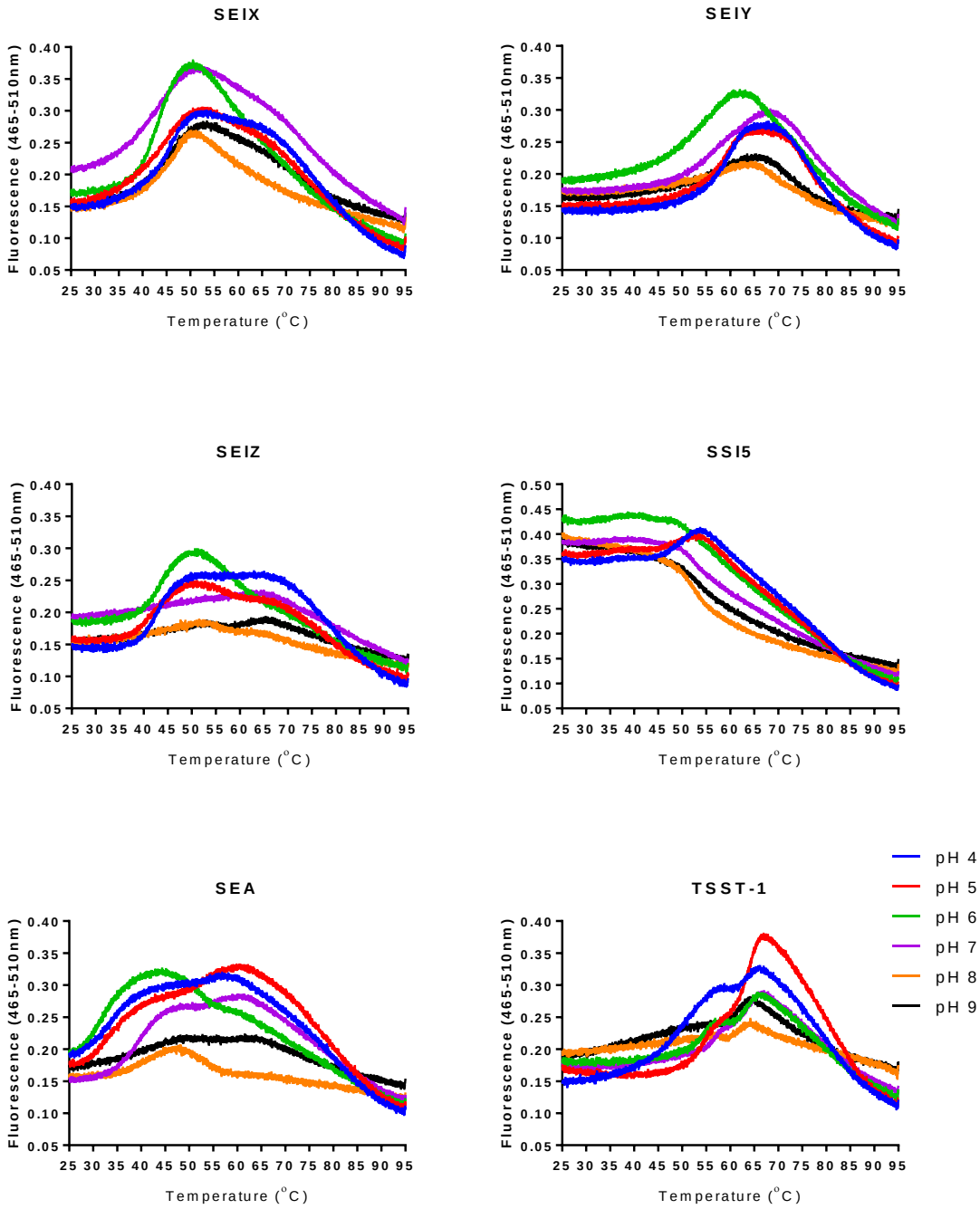


Figure 3.8 Staphylococcal SAgs exhibit a high level of tolerance to pH variation. Thermal shift analysis of staphylococcal proteins buffered with PBS at varied pH. Profiles were performed in triplicate and the mean fluorescence was plotted against temperature

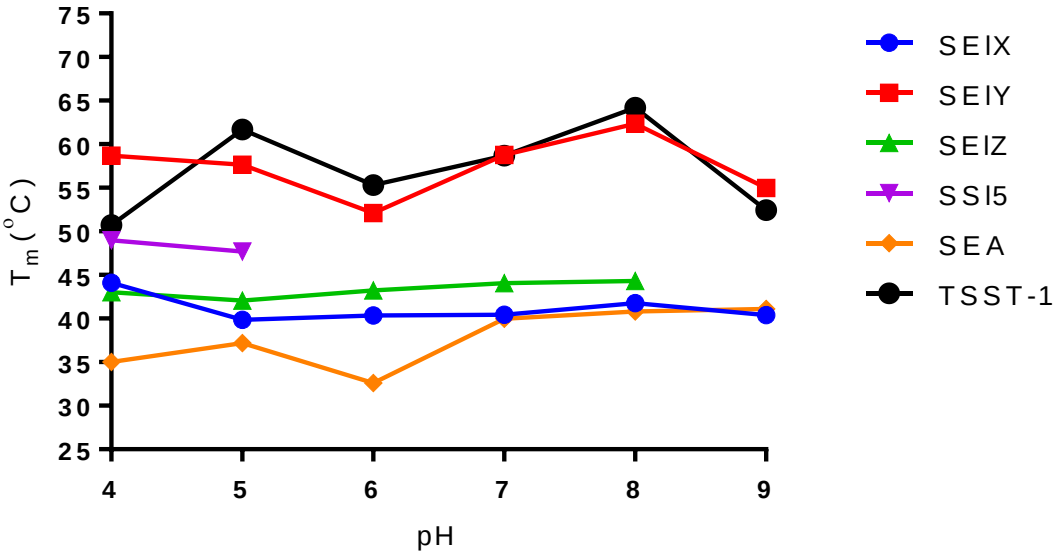


Figure 3.9 SAgs demonstrate limited variation in stability at different pH. Mean fluorescence spectra of SAg thermal shift profiles at different pH were used to determine the T_m (50% of protein unfolding) of each protein under each condition. If a T_m was not determinable due to degradation no data point was plotted. The T_m was plotted against pH.

3.3.6 SElZ demonstrates zinc-dependent binding.

Zinc ions can be utilised by SAgs to interact with the MHC class II complex (Spaulding et al., 2013). If zinc binds to SAgs it may alter the stability of the proteins and change the thermal shift profile. Here, 5 μM of recombinant protein was co-incubated with differing concentration of zinc sulphate and the divalent cation chelator EDTA. Zinc compounds will precipitate in neutral and alkaline solutions and in PBS so all profiles were obtained in 50 mM HEPES at pH 6. The thermostability profiles of TSST-1, SElX and SElY were unchanged between 10 μM to 0 μM of Zn^{2+} and for the protein incubated with EDTA (Fig 3.10). The thermal profiles of TSST-1, SElX and SElY demonstrate instability when incubated with the higher concentrations of zinc. Taken together these data are consistent with a lack of a biologically relevant interaction for TSST-1, SElX and SElY with zinc. SSl5 shows an inverse relationship between zinc and thermostability (Fig 3.10) as thermal profiles of SSl5 exhibited more rapid degradation when more zinc was added. The most stable SSl5 profile was exhibited when SSl5 was incubated with EDTA, which suggests that SSl5 is sensitive to the presence of divalent cations like zinc.

SEA has been demonstrated to interact with zinc during the interaction between MHC class II and the high affinity binding site on the protein (Hudson et al., 1995). Here we demonstrate that SEA stability increases as more zinc is added indicative of zinc binding. The thermostabilty profiles of SElZ are more varied exhibiting the highest level of stability at 1 μM of zinc. The second highest level of SElZ stability was exhibited by the protein suspended in EDTA. This suggests that SElZ binds to zinc but this interaction has an optimal concentration and can be destabilised by higher concentrations of the cation. This suggests that SElZ binds zinc cations and could be used by the protein to form dimers or bind to the MHC class II complex through the high affinity site.

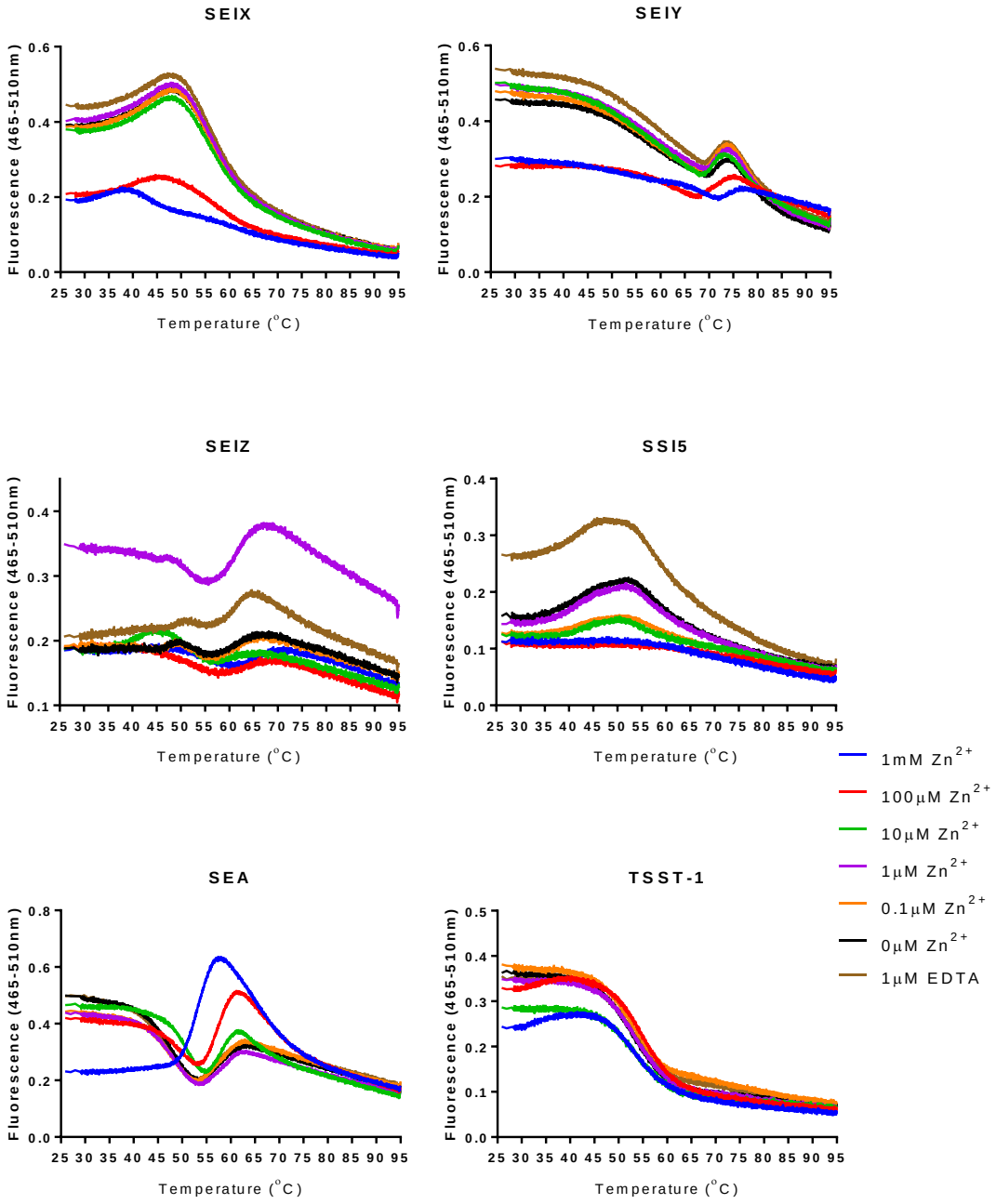


Figure 3.10 Zinc binds differentially to staphylococcal SAgs. Thermal shift experiments of different staphylococcal proteins co-incubated with different zinc sulphate concentration. Profiles were performed in triplicate and the mean fluorescence was plotted against temperature

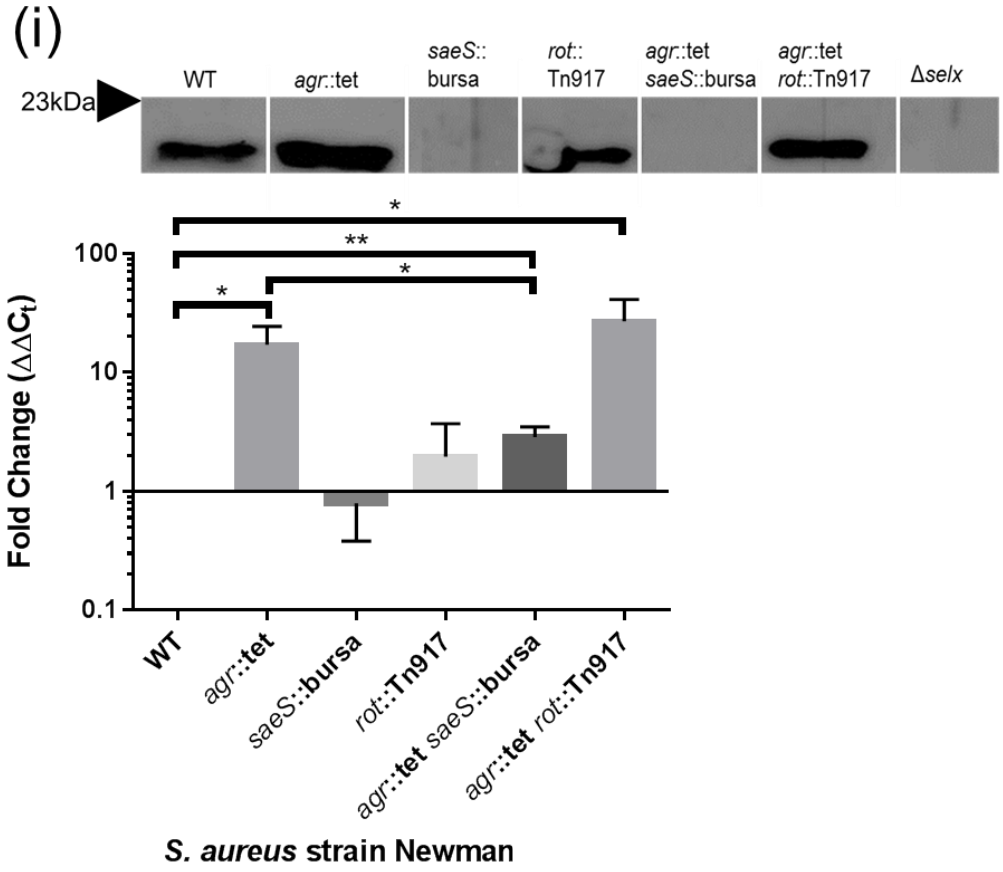
3.3.7 SElX and SElZ do not exhibit emetic activity.

SElX and SElZ exhibit a high level of stability and SElZ contains a cysteine loop, a characteristic of potential emetic activity. It is important to identify the potential to induce emesis as staphylococcal enterotoxins are a leading cause of food poisoning in humans (Hennekinne et al., 2012). Activity has traditionally been assessed in non-human primate models which are expensive and ethically questionable. Recently an alternative rodent model of emesis in the house musk shrew (*Suncus murinus*) has been developed (Hu et al., 2003). The animals were assessed for emesis following intraperitoneal administration of protein. 500 μg/ml of SElX and SElZ was injected into 4 and 5 animals respectively. Emesis was not observed after 3 h and no behavioural changes noted. The dose was increased to 1mg/ml for each protein and again no emesis or behavioural changes were observed demonstrating that these proteins are not emetic in this model. These experiments were performed at Hirosaki University, Japan, by Dr. Hisaya Ono and supervised by Prof. Akio Nakane. SElY was found to be emetic independently of this study (Ono et al., 2015).

3.3.8 Regulation of SElX expression is dependent on the *sae* locus.

The importance of SElX in a rabbit model of USA300-induced necrotizing pneumonia and abscess models demonstrates up-regulation of expression during infection (Malachowa et al., 2015, Wilson et al., 2011). In order to examine the regulation of expression of SElX, insertion mutants of selected global regulators were assessed for SElX gene and protein expression in strains Newman and USA300 LAC. Similar mutants for strains encoding SElY and SElZ were not available at this time so this analysis was not performed for these SAgs. Supernatants and bacterial cells from the two wild type *S. aureus* strains and 8 global virulence regulator mutants (Table 3.1) attenuated for expression of regulators *agr*, *saeRS* and *rot* were analysed by Western blot and qRT-PCR analysis for SElX expression (Fig

3.11). The expression of the *selx* gene was analysed at post-exponential phase (OD_{600}= 7.0 for Newman and OD_{600}= 4.0 for LAC) as this is the peak point of expression of *selx* previously reported in liquid media (Malachowa et al., 2011). Fold change data was calculated based on the transcription of *selx* from the wild type after the each data point was normalised using the internal control (16srRNA). Western blot analysis was performed on supernatants from bacterial cultures grown for 16 h. The disruption of the *agr* locus resulted in increased *selx* expression in strain Newman, a trend that was not significant in USA300 LAC (Fig 3.11), consistent with a strain-dependent role for *agr* as a repressor of *selx* expression. When the *rot* gene is disrupted there is no effect on *selx* transcription or protein expression suggesting this locus has no direct impact of SElX expression (Fig 3.11). In contrast, the loss of *sae* expression resulted in a massive reduction in levels of SElX protein for both strain Newman and USA300 LAC (Fig 3.11). In Newman the disruption of *sae* resulted in SElX being undetectable in both the single *sae* and double *sae* and *agr* mutants (Fig 3.11). In LAC SElX expression was greatly diminished in the *sae* insertion mutant (Fig 3.11). When the transcript analysis was performed on the *sae* mutants it was found that *selx* transcripts were significantly lower in the *sae* mutant of LAC and reduced in Newman, although not significantly (Fig 3.11). However there was a significant reduction in *selx* transcripts when comparing the *agr sae* combination mutant compared to the *agr* only insertion mutant of Newman. Taken together these data demonstrate that the *sae* locus is a regulator of SElX expression in *S. aureus.*


(i)
WT
agr::tet
saeS::
bursa
rot::
Tn917
agr::tet
saeS::bursa
agr::tet
rot::Tn917
ΔselX
23kDa
Fold Change (ΔΔCt)
100
10
1
0.1
*
**
*
*
WT
agr::tet
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rot::Tn917
agr::tet saeS::bursa
agr::tet rot::Tn917
S. aureus strain Newman

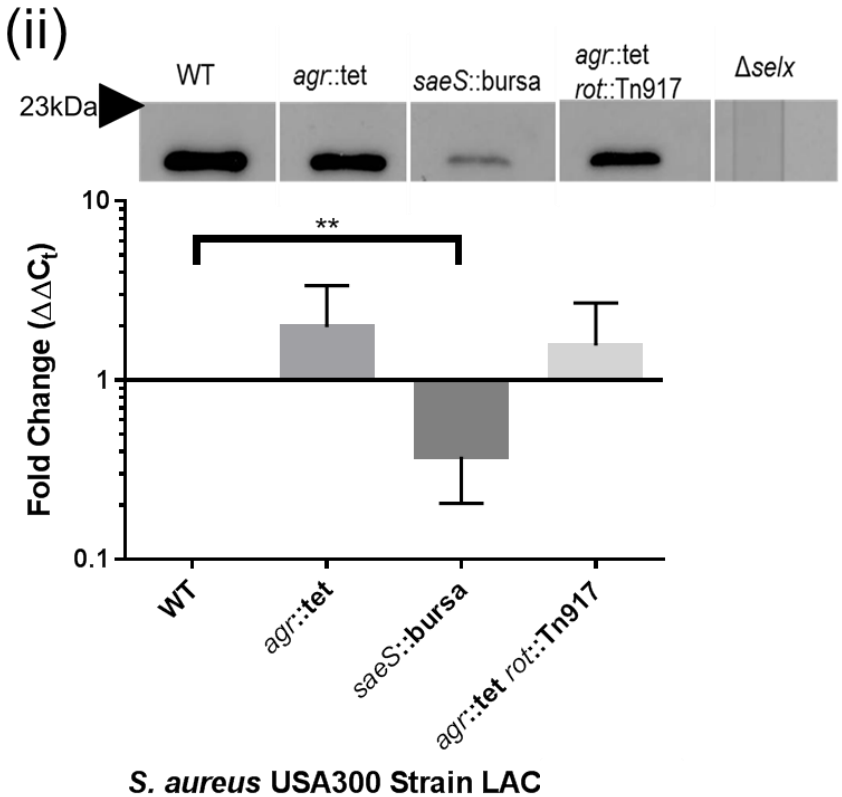

(ii)
WT
agr::tet
saeS::bursa
agr::tet
rot::Tn917
ΔselX
23kDa
Fold Change (ΔΔCt)
10
1
0.1
**
WT
agr::tet
saeS::bursa
agr::tet rot::Tn917
S. aureus USA300 Strain LAC

Figure 3.11: Expression of *selx* is regulated by the two component sensor system *saeRS*. Expression analysis of global regulator insertion mutants from *S. aureus* strains Newman (i) and USA300 LAC (ii) by western blot and qRT-PCR analysis. Western blots were performed on concentrated overnight supernates and detected using anti-SElX IgY. RNA was purified from bacteria grown to OD_{600} = 7.0 for strain Newman and OD_{600} = 4.0 for USA300 LAC and analysed by SYBR™ green qRT-PCR. Data was normalised to fold change from the wild type ($\Delta\Delta C_t$) and the results shown are the means of four independent experiments ± the standard deviation of the mean.

3.4 DISCUSSION

In this study the SAgs SElX, SElY and SElZ all were shown to contain motifs found in other SAg which have been demonstrated to be involved in SAg function. The phylogenetic analysis demonstrated that *selx* and *sely* are more closely related to the SSl-proteins than they are to the majority of SAgs. The predicted models all suggest SElX, SElY and SElZ exhibit the classical two domain arrangement and encode residues in the expected regions of the protein that can mediate TCR and MHC class II binding. All three SAgs were found to encode a variant of the outside-in signalling motif which is found in all other SAgs (Brosnahan and Schlievert, 2011). This motif appears to be a critical component of SAg structure, in a similar fashion to the TCR and MHC class II binding sites (Brosnahan and Schlievert, 2011, Stach et al., 2014). The alignment of this motif from different SAgs shows that the dodecapeptide is diverse in amino acid sequence (Fig 3.4) and may mediate specificity to different cell surface receptors. The motif has been implicated in transcytosis of SAgs like TSST-1 and may also be the binding site for tertiary binding partners involved in T-cell activation such as CD28 and CD40 which have been implicated in cytokine activation by SAgs (Arad et al., 2011, Brosnahan and Schlievert, 2011, Stach et al., 2014).

The sequence analysis of SElY and SElZ demonstrates that these SAgs are distributed among *S.aureus* strains at a level comparable to other staphylococcal SAgs (5 % to 25 %) (Fraser and Proft, 2008b, Spaulding et al., 2013). Both SAgs were found in *S. aureus* strains isolated from a range of host species including humans, cattle and rabbits. For both SAgs a number of allelic variants were identified and further work should be conducted to examine their distribution across the *S. aureus* population and the potential functional significance of the allelic variation.

SElX is predicted to exhibit a unique structure for a staphylococcal SAg in that it has a truncated N-terminal domain made up of loop structures (Wilson et al., 2011). This is

contrary to the CD spectra obtained which suggests that the predominant secondary structure in the protein is α-helices. The prediction software used to prepare the model of SElX utilised the crystal structure of TSST-1 and other SSl-proteins as templates. Given the vast difference in the CD spectra of SElX, TSST-1 and SSl7 it suggests the model for SElX is inaccurate and that the structure of SElX maybe unique. The OB fold is the location of the low affinity binding site for MHC class II. As SElX does not to bind to zinc and given the novel structure of the OB domain, it remains unclear how SElX interacts with the MHC class II complex. This data may be linked to the lower potency of SElX relative to other SAgs.

Further analysis of SElX demonstrated that it contains a sialic acid binding motif, a common feature of many SSl-proteins. This protein motif mediates the binding of many SSl-proteins to neutrophils and maybe the site of interaction between SElX and neutrophils previously reported (Fevre et al., 2014). This motif is identical in 11 of the 17 identified allelic variants of SElX with the six variants that are not identical demonstrateing only a single amino acid difference in position 12 (Fig 3.4), a residue previously demonstrated to interact with sLEX in crystallography studies (Hermans et al., 2012). Unlike the three other residues demonstrated to bind to sLEX, position 12 varies among the SSl-proteins and so far histidine, aspartic acid and asparagine have all been identified at this site in different SSl-proteins suggesting a possible role in tropism to specific sugars or glycoproteins. The conservation of the sialic acid binding motif in all variants of SElX associated with strains infecting multiple hosts suggests that sugar interactions may be an important general function for SElX in the pathogenesis of human and animal species.

The thermal shift assay demonstrated that four (SElX, SElY, SSl5 and TSST-1) of the six proteins tested did not interact with zinc and suggest that SElX and SElY do not encode the MHC class II high affinity binding site and likely interact with MHC class II through the low affinity site only, similarly to TSST-1 (Spaulding et al., 2013). SEA demonstrates an increase in stability when the zinc ligand is added, which may suggest why

SEA exhibits such a low tolerance to pH variations, as the assay was performed in the absence of zinc. For SElZ the data from the zinc binding assays were not conclusive as 1μM of zinc increased the stability of SElZ, but other concentrations either conferred a limited increase in stability or destabilised the protein (Fig 3.12). Another possibility for the limited zinc binding observed in SElZ is the presence of the HIS-tag. This motif is controlled for as it is presenet in all the proteins tested but it could still contribute to an increase in zinc binding. Given that SElZ is predicted to encode two zinc binding sites, it is likely that there is a genuine interaction with zinc and binding to MHC class II can occur via the high affinity binding site.

The data obtained in this study suggests that SElZ exhibits the structural and functional traits of a classical group II SAg. The T-cell proliferation assay demonstrated that this protein was also a potent mitogen for human T-cells. It was also shown to exhibit high level of stability to variation in temperature and pH. During the stability study it exhibited a biphasic degradation profile similar to that observed for SEA. SEA contains the cysteine loop structure in the OB domain of the protein which SElZ is also predicted to contain. The biphasic profile seen may be indicative of this structure in both SEA and SElZ as the first phase of unfolding is associated with regions of the protein not associated with the loop structure. As the cysteine loop is a more stable structure, a second peak in fluorescence is observed as the temperature reaches a level high enough for degradation of the loop. Despite exhibiting a stable structure and containing a cysteine loop, SElZ was non-emetic in the house musk shrew model. This model has been shown to be able to demonstrate emesis for potent enterotoxins like SEA but may behave inconsistently for less potent emetic proteins (Omoe et al., 2013). It is feasible that SElZ is emetic at high concentrations in humans, and to investigate this SElZ should be examined in a non-human primate model.

SElY has been demonstrated to be a highly stable protein in this study and is predicted to share a structure similar to TSST-1. Further, SElY is emetic in the house musk

shrew model (Ono et al., 2015). This suggests that SElY is capable of mediating food poisoning in humans. Like SElX there is a discrepancy between the predicted model and the similarity between the CD spectra of SElY and TSST-1. The SElY spectrum is indicative of more α-helical structure than that predicted in the model and crystallography studies should be performed to understand the structure of this SAg. SElY is phylogenetically related to SET which uniquely does not have a Vβ T-cell profile and may induce T-cell proliferation independent of the Vβ region of the TCR. SElY is a potent T-cell mitogen in humans but the Vβ profile is still to be determined and it may prove to behave in a similar way to SET.

In the case of both SElX and SElY the CD data varied significantly from the predicted model of both proteins. In both cases the templates used to prepare the model were TSST-1 and a number of SSl-proteins including SSl7. The CD spectrum of both SSl7 and TSST-1 were found to be completely different from that seen for SElX or SElY, there was strong indication for the presence of the random coil and less α-helices in the secondary structure of TSST-1. In SSL7 the CD spectra generated gave a relatively weak signal compared to the other proteins examined but the spectra was more suggestive of a secondary structure made up of an equal amounts of α-helices and β-sheets. SElX and SElY CD spectra suggest two protein structures predominanted by α-helices and may suggest a novel arrangement compared to other SAgs. The presence of the outside-in signalling motif suggests the central α-helix is likely still present and may still separate the protein into two separate domanins as seen in other SAgs. The structural analysis of these proteins highlights the limitations of protein modelling and that the accuracy is highly dependent on the availability of crystal structures from closely related proteins. The group I superantigens are a disparate group and sequence identity within this group is low (<20%) and it is unsurprising that the models generated from using TSST-1 and other less closely related proteins such as SSl-proteins do not necessarily reflect the reality.

The expression data demonstrates that *selx* expression is globally regulated by both the *agr* and *sae*R/S locus. The *agr* locus is acting as a repressor of *selx* expression as RNA transcripts were significantly increased in the Newman strain when *agr* was disrupted. To date many SAgs have been shown to be positively regulated by the *agr* locus suggesting that *selx* is not employed by *S. aureus* during infection in the same way as other SAgs. The *saeR/S* locus is an activator of *selx* expression as the disruption of this locus saw a very large reduction in *selx* expression in both backgrounds tested. Like other genes regulated by the *saeR/S* locus *selx* was found to encode a consensus sequence in the promoter region of the gene which SaeR can bind to directly and up regulate expression (Nygaard et al., 2010). The *saeR/S* locus has been implicated in the response of *S. aureus* to phagocytic signals although it is not clear precisely which signals can activate the pathway and there is evidence to suggest the pathway can be activated differentially by different signals (Geiger et al., 2008, Voyich et al., 2009). The *saeRS* regulon included a wide number of different virulence determinants including the SSl-proteins 5 and 7, CHIPS and FLIPr (Nygaard et al., 2010). This study has shown that *selx* is regulated by a locus that responds to phagocytic signals and activates expression of virulence determinants that have been shown to contribute to the evasion of phagocytes. Taken together with the presence of the sialic acid binding motif within SElX there is a case to further explore if SElX can interact with neutrophils and other leukocytes to disrupt their function.

Chapter 4

Superantigen Binding to Leukocytes

4.1 INTRODUCTION

So far in this study we have demonstrated that SElX contains characteristic motifs of both SAgs and SSl-proteins. The regulation of *selx* also suggests it may be expressed as part of the *S. aureus* response to the innate immune system given its co-regulation with other innate immune modulators such as CHIPS, SCIN and the SSl-proteins (Nygaard et al., 2010, Rooijakkers et al., 2006). It has already been demonstrated that SElX can interact directly with human neutrophils and at very high concentrations interact with the glycoprotein PSGL-1 (the same target as SSl5) (Fevre et al., 2014, Bestebroer et al., 2007). This interaction is unlikely to be the only target of SElX on neutrophils as the concentration required to bind PSGL-1 is at a physiologically irrelevant concentration (Fevre et al., 2014). Taken together these data suggests SElX may act as innate immune modulator by binding to neutrophils.

SElX was found in the previous chapter to contain a predicted sialic acid binding motif, a motif found in the SSl-proteins, SSl4, SSl5, and SSl11 which coordinates the binding of sialic acid decorating proteins on the surface of neutrophils (Baker et al., 2007, Chung et al., 2007, Hermans et al., 2012). SSl3 also contains the sialic acid binding motif but binds to its target on the neutrophil (TLR2) independent of the glycan binding motif demonstrating that SSl-proteins can interact with neutrophil through mechanisms independent of sialic acid (Koymans et al., 2015). The characterisation of neutrophil binding SSl-proteins has demonstrated that these proteins have a range of targets on the surface of the neutrophil (Table 1.2). The binding of SSl-proteins to the surface of a neutrophil can directly antagonise the target and block its function (Bestebroer et al., 2007), but binding may also lead to internalisation of the protein which could have an intracellular function (Chung et al., 2007).

The SAgs TSST-1 and SElY also have been shown to be phylogenetically more closely related to the SSl-proteins than the majority of SAg and therefore warrant further study for any potential innate immune inhibitory functions. Potential neutrophil interactions should also be analysed with SElY and TSST-1 to determine if the SAg-neutrophil interaction is a property limited to SElX. Here we will characterise the binding of SElX and other SAgs to neutrophils by:

- Comparing the binding of SElX and other SAgs to leukocytes from different mammalian species
- Using site directed mutagenesis to determine the residues involved in SElX binding to neutrophils
- Determining which host cell surface receptors are involved in SElX neutrophil interactions

4.2 MATERIALS AND METHODS

4.2.1 Neutrophil isolation from different mammalian species

Human PMNs and PBMC were isolated from human blood as outlined in section 2.16. Murine bone marrow derived PMNs were isolated from femurs of 10 to 16 week old BalbC mice, as described previously (Mocsai et al., 2003). Femurs were harvested after sacrifice and washed in murine-preparation buffer (HBSS (Mg^{2+} and Ca^{2+} free) (Gibco, UK) supplemented with 20 mM Na-HEPES (Fisher Scientific, UK) (pH 7.4) and 0.5% FCS (Sigma Aldrich, UK)) to remove any muscle tissue. Bone marrow was flushed from the femurs with excess murine-preparation buffer, homogenised and then passed through a 70 μm cell strainer (BD bioscience, Oxford, UK) to remove bone fragments. The suspension was then pelleted by centrifugation at 400 x g for 5 min and re-suspended in 10 ml of 0.2 % (w/v) NaCl to lyse red blood cells for 30 s to 40 s. Osmolarity was restored with 10 ml 1.6% (w/v) NaCl and the cells were again pelleted by centrifugation at 400 x g for 5 min. Percoll (GE, healthcare, Buckinghamshire, UK) was mixed with murine-preparation buffer to prepare a 62.5 % (v/v) gradient and 5 ml was added to a 15 ml conical tube. The cells were re-suspended in 5 ml murine-preparation buffer and layered onto the 62.5 % Percoll. The suspension was then centrifuged for 30 min at 1000 x g with the centrifuge brake off. At the end of the centrifugation the neutrophil pellet was harvested (diffuse layer found in the middle of the 62.5 % percoll). The pellet is washed twice with murine-preparation buffer and then re-suspended in 1 ml of assay buffer for counting with the TC20 automated cell counter (Biorad, Hemel Hempstead, UK) and re-suspended to a concentration of 1 x 10^7 cells/ml. Neutrophil phenotype was confirmed using Lys6C and Lys6G staining by incubating the samples of the isolated neutrophils individually with 1:500 dilutions of with Rat anti-Lys6C PerCP-Cy5.5 (BD Bioscience, Oxford, UK) and Rat anti-Lys6G PE-Cy7 (BD Bioscience, Oxford, UK) for 30 min on

ice before the cells were analysed by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lanes, NJ). Phenotype was confirmed by a high fluorescent signal for Lys6G and Lys6C.

Bovine PMNs were isolated from Holstein-Friesian cattle aged 18 to 36 months via jugular vein puncture (6 ml 10 x PBS/EDTA (100 mM KH_2PO_4, 9 % (w/v) NaCl, and 2 mg/l EDTA in sterile H_2O adjusted pH to 7.4) anticoagulant used for 50 ml of blood). Animals were reared indoors and maintained on a ration of hay and concentrates. PMNs were isolated from bovine blood using a protocol described previously (Siemsen et al., 2007). 50 ml blood was pooled into a conical 50 ml polypropylene centrifuge tube (BD bioscience, Oxford, UK) and centrifuged at 740 x g for 10 min at room temperature with low brake. The upper plasma layer and buffy coat (found at the plasma–red blood cell interface) were removed and the remaining red blood cell layer was split equally between 5 polypropylene centrifuge tubes. Red blood cells were lysed by adding 10 ml of sterile H_2O to each tube and gently inverted for 20 s at room temperature. Osmolarity was restored by adding 1 ml of 10 % (w/v) NaCl solution to each tube. The leukocyte pellet was recovered by centrifugation at 585 x g for 10 min at room temperature with low brake. The cell pellets were pooled and re-suspended in 10 ml of HBSS (Mg^{2+} and Ca^{2+} free). Histopaque gradients were prepared by first layering 15 ml of Histopaque 1077 (Sigma-Aldrich, UK) onto 15 ml Histopaque 1077/1119 solution (1:1 solution of Histopaque 1119 and Histopaque 1077 (Sigma-Aldrich, UK)) in a conical 50 ml centrifuge tube. The leukocyte suspension was layered on top of the Histopaque and centrifuged at 440 x g for 25 min at room temperature with no brake. The neutrophil pellet was harvested and washed by re-suspending the cells in 50 ml of HBSS and centrifuging at 585 x g for 10 min at room temperature. The cells were then counted with a TC20 automated cell counter (Biorad, Hemel Hempstead, UK) and re-suspended to a concentration of 1 x 10^7 cells/ml in the desired assay buffer.

Rabbit PMNs were isolated from whole rabbit blood (using ACD (see section 2.16) as an anticoagulant) purchased from the National Transfusion Centre, Moredun Institute, Midlothian, UK. Leukocytes were isolated using an equal volume of red blood cell (RBCs) lysis buffer (10 x buffer contained; 155 mM NH_4Cl, 10 mM $KHCO_3$ and 100 μM EDTA in ddH_2O). RBCs were lysed in 1x Buffer for 10 min at 37°C and the remaining cells were pelleted at 400 x g for 10 min and washed with HBSS (Mg^{2+} and Ca^{2+} free) (Gibco). This was repeated until the pellet was clear of red cells and the cells were then counted with a TC20 automated cell counter (Biorad, Hemel Hempstead, UK) and re-suspended to a concentration of 1 x 10^7 cells/ml in the desired assay buffer.

4.2.2 Protein expression and purification

Plasmid pET15b and pQE30 constructs containing SAg genes previously prepared in Chapter 3 were used to prepare recombinant proteins (Table 3.3). Protein expression was induced as outlined in section 2.13.1. All recombinant proteins were purified using the native purification protocol outlined in section 2.13.2 except SElX and SSl5. SElX was purified using the denaturing protocol outlined in section 2.13.2 followed by a second purification step using IEC also described in section 2.13.2. SSl5 was purified with a hybrid purification protocol; induced cells were lysed and bound to the affinity column with denaturing protocol buffers (Table 2.1) using the protocol described in section 2.13.2 and then washed with the denaturing wash buffer as described in 2.13.2. Protein was then refolded on the column by adding native protocol wash buffer over a gradient (30 min to 100 % at a flow rate of 1 ml/min). The protein was eluted with native protocol elution buffer.

4.2.3 Cell binding experiments

For binding of recombinant proteins to leukocytes, neutrophils (5×10^6 cells/ml) and PBMCs (5×10^6 cells/ml) were incubated with increasing concentrations of 6 x HIS-tagged recombinant proteins in RPMI 1640 (Gibco, UK) and 0.05 % HSA (Human serum albumin) (Sigma-Aldrich, UK) (RPMI-HSA) for 30 min on ice. For lymphocyte binding experiments the cells were also co-incubated with lymphocyte subset–specific antibodies including anti-CD4, -CD8, and, -CD19 (PE labelled) (BD bioscience, Oxford, UK). Cells were washed with RPMI-HSA and pelleted at 400 x g for 10 min at 4°C. Binding of the proteins was detected with a FITC-labelled monoclonal mouse anti-HIS tag monoclonal antibody (LS Bioscience, WA, USA), antibodies were incubated with cells for 30 min on ice, washed with RPMI-HSA and pelleted at 400 x g for 10 min at 4°C. Following washing, cells were re-suspended in 200 µl of RPMI-HSA and leukocyte populations were identified based on forward and sideway scatter on a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lanes, NJ), and fluorescence was measured. For neuraminidase experiments isolated leukocytes (2×10^6 cells/mL) were pre-treated with 0.2 U/ml neuraminidase (from Vibrio *cholerae*; Sigma-Aldrich, UK) at 37°C for 45 minutes at pH 6.0, prior to washing and subsequent incubation.

4.2.4 Site-directed mutagenesis

Site-directed mutagenesis was performed to replace amino acids in the sequence of SElX by introducing mutations into the pET15b::*selx2* construct using PCR reactions with oligonucleotide primers listed in Table 4.1. The reactions were performed using the PfuUltra II Fusion HS DNA polymerase (Agilent Technologies, UK) as described in section 2.7. Primers were used at a final concentration of 250 nM along with 2 mM dNTPs (Promega, Hampshire, UK). Vector DNA was added to a final concentration of 5 to 30 ng and the PCR

reactions were performed on a MyCycler™ thermal cycler (Biorad, Hemel Hempstead, UK); 1 cycle at 95°C for 2 min, 30 cycles of 95°C for 20s, 50°C for 20s and 72°C for 90s, followed by a final extension of 3 min at 72°C. Following the PCR amplification *Dpn*I endonuclease (New England Bioscience, Herts, UK) was added to a final concentration of 0.8 U/µl, the reaction was then incubated for 1 h at 37°C followed by an enzyme deactivation step of 10 min at 65°C. Following digestion, 1µl of the amplified vector product was transformed into *E. coli* Solopack™ cells from the Strataclone blunt cloning kit (Aglient technologies, UK). The ligated mixture was transformed into the defrosted competent cells and pre-incubated on ice for 20 min, followed by a heat shock at 42°C for 45s before returning to ice for 2 min. 250 µl of pre-warmed LB was added to the cells to recover for an hour at 37°C before 100 µl of the bacterial suspension was plated onto LB agar containing 100 µg/ml of ampicillin.

Plates were grown overnight at 37°C and screened for transformed colonies which were randomly selected for Sanger sequencing. Briefly, transformed colonies were grown overnight in liquid culture and the plasmids were purified using the Qiaprep miniprep kit (Qiagen, Manchester, UK) according to the manufacturer's specifications. Plasmid constructs were sequenced by Edinburgh Genomics Sanger sequencing service (University of Edinburgh, UK). When the mutations were confirmed the plasmid constructs were transformed into BL21 DE3 *E. coli* cells for protein expression and purification (see section 2.13). To ensure that protein folding and stability was not affected by the mutagenesis, CD and thermal shift analysis was carried out.

Table 4.1 Oligonucleotide Primers used for site directed mutagenesis of pet15b::*selx2*.

Primer Name	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Nucleotide Mutation[a]	Amino Acid Exchange
E153	GTCATAAATACAAAAGATGGTGGTAAATATACATT AGCTTCGCATAAAGAGCTACAAAAAGATAGGG	CCCTATCTTTTTGTAGCTCTTTATGCGAAGCTAA TGTATATTTACCACCATCTTTTGTATTTATGAC	499 GAG>GCT	E153A
K156	GATGGTGGTAAATATACATTAGAGTCGCATGCAGA GCTACAAAAAGATAGGGAAAAT	ATTTTCCCTATCTTTTTGTAGCTCTGCATGCGACT CTAATGTATATTTACCACCATC	508 AAA>GCA	K156A
Q159	GAGTCGCATAAAGAGCTAGCAAAAGATAGGGAAAA TGTAAAAA	TTTTTACATTTTCCCTATCTTTTGCTAGCTCTTTA TGCGACTC	517 CAA>GCA	Q159A
D161	GAGTCGCATAAAGAGCTACAAAAAGCAAGGGAAAA TGTAAAAATT	AATTTTTACATTTTCCCTTGCTTTTTGTAGCTCTT TATGCGACTC	524 GAT>GCA	D161A
EKQD	GGTGGTAAATATACATTAGCTTCGCATGCAGAGCTA GCAAAAGCAAGGGAAAAT	ATTTTCCCTTGCTTTTGCTAGCTCTGCATGCGAA GCTAATGTATATTTACCACC	499 GAG>GCT	E153A
			508 AAA>GCA	K156A
			517 CAA>GCA	Q159A
			524 GAT>GCA	D161A

[a] Positions relative to *selx2* allele

4.2.5 Circular Dichroism analysis

Far UV CD spectra of protein samples was recorded in 0.1 x PBS (pH 7.3) using the Jasco J-710 (Japan Spectroscopic Co. Ltd, Japan). Spectra of the proteins, between a concentration of 0.4 to 1 mg/ml, were recorded between 190 nm to 250 nm using a cuvette with a path length of 0.05 mm. For analysis the precise molar concentration was determined for each protein and used in the Jasco software to normalise each protein spectra.

4.2.6 Thermal shift analysis

To monitor protein unfolding, the fluorescent water sensitive dye Sypro orange (Life Technologies, UK) was employed. The thermal shift assay was conducted in the Lightcycler 480 (Roche, West Sussex, UK), the melt curve function of this system was used and a melt curve was determined from 25°C to 95°C. 25µl of 5 µM recombinant protein was incubated with Sypro orange dye diluted 1/125 of the original stock dye, with heating from 25°C to 95 °C at a heating rate of 0.1°C/sec. The fluorescence intensity was measured with excitation/emission at 465/510 nm.

4.2.7 Affinity precipitation and mass spectrometry analysis

This analysis was performed at the New York University Medical Centre by Dr David James and supervised by Prof. Victor Torres. Detergent-solubilized proteins from primary human neutrophils were incubated with 6 x HIS-tagged SElX and interacting proteins were purified using Ni-NTA resin (Qiagen, Manchester, UK). Proteins eluted from Ni-NTA resin were separated on a precast 4 % to 15 % gradient SDS-PAGE gel (Bio-Rad, Hemel Hempstead,

UK) and stained with SYPRO-Ruby (Life Technologies, UK). Gels were imaged and the lanes were excised for trypsin digestion, protein extraction and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

The gel was de-stained with 1:1 solution of 10 mM ammonium bicarbonate and 100 % (v/v) acetonitrile solution followed by 100 % (v/v) acetonitrile. The solvent was removed and the gel pieces were covered with trypsin digestion buffer (13 ng/µl trypsin (porcine sequencing grade (Promega, Hampshire, UK)) in 10 mM ammonium bicarbonate containing 10% (v/v) acetonitrile) and the buffer was allowed to saturate for 2 h on ice. Digestion was carried out overnight at 37°C.

The protocol for protein extraction is as follows; slurry of R2 20 µm Poros beads (Life Technologies, UK) in 5 % (v/v) formic acid and 0.2 % (v/v) trifluoroacetic acid (TFA) was added to each sample at an volume equal to that of the ammonium bicarbonate added for digestion, followed by shaking at 4˚C for 3 h. The beads were loaded onto equilibrated C18 ziptips (Millipore) by centrifugation for 30 s at 6000 rpm. Gel pieces were rinsed three times with 0.1 % TFA and each rinse was added to its corresponding ziptip followed by centrifugation. The extracted porous beads were further washed with 0.5 % (v/v) acetic acid and peptides were eluted by the addition of 40 % acetonitrile in 0.5% acetic acid followed by the addition of 80 % (v/v) acetonitrile in 0.5 % (v/v) acetic acid. The organic solvent was removed using a SpeedVac concentrator and the sample reconstituted in 0.5 % (v/v) acetic acid.

Samples were ready for LC-MS/MS analysis and 1/3 of each sample was analysed individually by Liquid chromatography separation connected with MS using the autosampler of an EASY-nLC 1000 (Thermo Scientific, USA). Peptides were gradient eluted from the column directly to Q Exactive mass spectrometer using a 1 h gradient (Thermo Scientific, USA). High resolution full MS spectra were acquired with a resolution of 70,000, an

automatic gain control (AGC) target of 1e6, with a maximum ion time of 120 ms, and scan range of 400 to 1500 m/z. Following each full MS twenty data-dependent high resolution, higher-energy C-trap dissociation (HCD) MS/MS spectra were acquired. All MS/MS spectra were collected using the following instrument parameters: resolution of 17,000, AGC target of 5e4, maximum ion time of 250ms, one microscan, 2 m/z isolation window, default charge state of +3, fixed first mass of 150 m/z, and normalised collision energy (NCE) of 27. MS/MS spectra were searched using a Uniprot Human database using Sequest within Proteome Discoverer (Thermo Scientific, UK).

4.2.8 Enzyme-linked immunosorbent assay (ELISA) validation of protein-protein interaction

96-well ELISA plates (Maxisorb; Nunc, Thermo, UK) were coated with 10 µg/ml recombinant SElX WT, SElX EKQD-A, SSL5 or SSl7 in carbonate/bicarbonate buffer (50mM Na_2CO_3 and $NaHCO_3$) diluted 1/10 in PBS. The plates were incubated overnight at 4°C. Plates were washed with PBS-0.05 % (v/v) Tween-20 (Sigma-Aldrich, UK) and blocked with PBS-0.05 % (v/v) Tween-20 and 8% (w/v) skimmed milk powder (microbiology grade; Sigma-Aldrich, UK) for 1 h at 37°C. Plates were washed with PBS-0.05 % (v/v) Tween20 and incubated with different **c**oncentrations of PSGL-1-HIS, CD50-HIS and CD16b-HIS (all purchased from Life Technologies, UK) for 1 h at 37°C. Bound HIS-tagged proteins were detected using mouse monoclonal antibodies for each target protein (AbD Serotec, Biorad, UK) followed by a peroxidase-conjugated rabbit-anti-mouse monoclonal antibody (Abcam, Cambridge, UK). Peroxidase activity was detected with TMB (Sigma-Aldrich, UK) for 40 s and the reaction was stopped using 1M H_2SO_4. Absorbance was measured at 450 nm using Synergy HT plate reader (BioTek, Vermont, USA).

4.3 RESULTS

4.3.1 Characterisation of binding of SAgs to human cells.

In order to test the hypothesis that SAgs can bind to human leukocytes the four SAgs; SElX, SElY, SElZ and TSST-1 were incubated with human leukocytes isolated from blood taken from healthy donors. Binding was detected by determining the fluorescent signal from a (FITC labelled) anti-HIS tag antibody so binding of all recombinant proteins tested can be compared directly. The SSl-protein SSl5 has previously been demonstrated to bind to neutrophils and monocytes previously (Bestebroer et al., 2007) and here we could reproduce those findings at concentrations below 20 nM of protein. SSl7 did not demonstrate any cell binding. Importantly SElX bound to human neutrophils and monocytes with a similar affinity and protein concentration to SSl5 (Fig 4.1). At higher concentrations SElX exhibited a higher binding signal than SSl5 when incubated with neutrophils, suggesting that SElX may have a higher affinity for human neutrophils than SSl5. In contrast the binding curves of SSl5 and SElX for monocytes was consistent with a similar affinity. Also observed was a lower affinity interaction between SElY and human neutrophils and monocytes, which was detectable above a 100 nM protein concentration. There was a higher signal for SElY-monocyte binding versus neutrophils binding which may indicate SElY has a higher affinity for monocytes. The SAgs SElZ and TSST-1 did not demonstrate binding to any of the human cells tested.

A variable low affinity interaction was observed between SElX and lymphocytes in some experiments, and co-staining of SElX and lymphocyte markers revealed (Fig 4.2) that SElX binds to $CD4^+$ and $CD8^+$ T-lymphocytes subsets but not to B-lymphocytes (CD19). SElY, SElZ and TSST-1 did not demonstrate any binding activity for lymphocytes (Fig 4.1).

4.3.2 SElX interacts with PMNs from different mammalian species.

S. aureus can infect a broad range of mammalian hosts (Fitzgerald, 2012). To determine if the observations made in the human cell binding analysis could be extended to different host species, neutrophils from 3 additional mammalian species (murine, laprine and bovine) were analysed for binding to the staphylococcal SAgs tested earlier on human neutrophils. The species were selected as *S. aureus* is a major pathogen of cattle, and both rabbits and mice are commonly used as models for *S. aureus* infections. In this experiment, only the $SElX_2$ recombinant protein was tested, which is an allele found only in human isolates of *S. aureus* (Fig 4.3). Murine cells demonstrated a high affinity for SElX, which was greater than that observed for human cells. Bovine and laprine cells exhibited lower binding but SElX and SSl5 binding could still be observed at a concentration below 100 nM (Fig 4.3). Overall SElX was capable of binding with PMNs isolated from the three species tested with an affinity that exceeded that of SSl5. SElY was the only other SAg tested that demonstrated binding to human neutrophils, but activity was not observed for cells from the additional mammalian species tested (Figure 4.3).

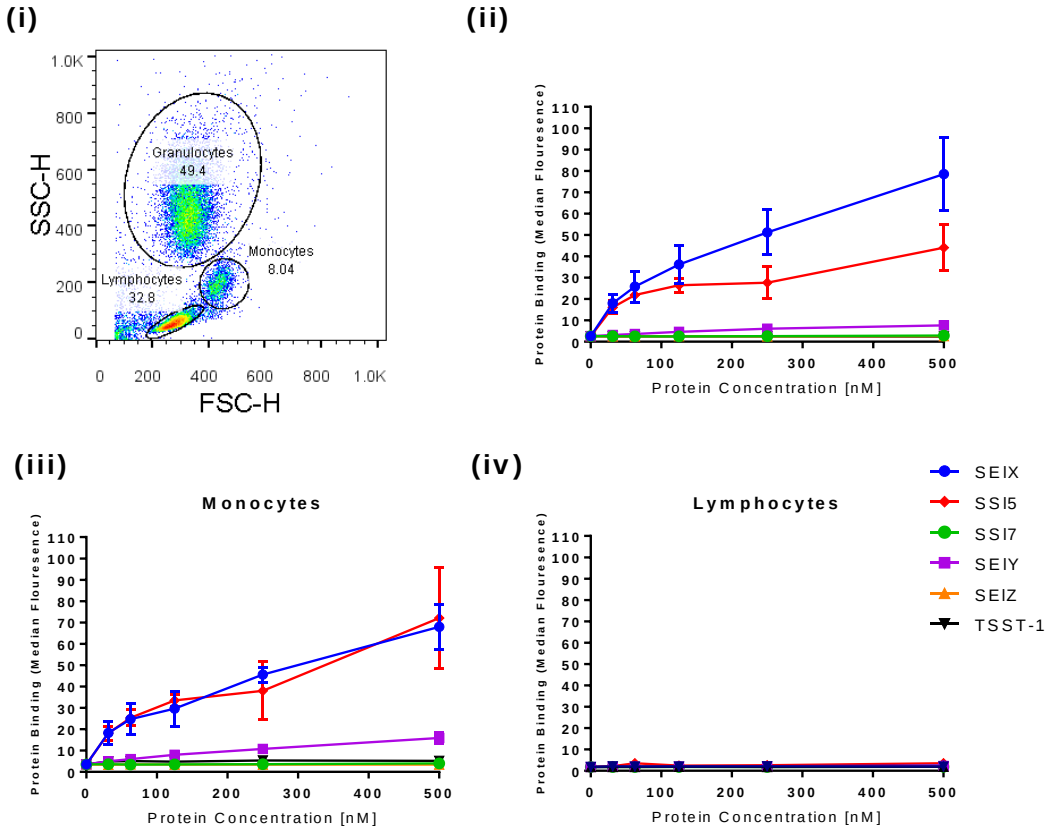


Figure 4.1 SElX and SElY bind to human monocytes and neutrophils. Flow cytometry analysis of binding of recombinant staphylococcal proteins to isolated human white blood cells. Binding to human cells was detected using Mouse anti-HIS-FITC IgG binding to the 6 x HIS-tag on the recombinant proteins. Mean median fluorescence of three donors is shown ± standard error of the mean. SSl5 and SSl7 were used as positive and negative controls respectively.

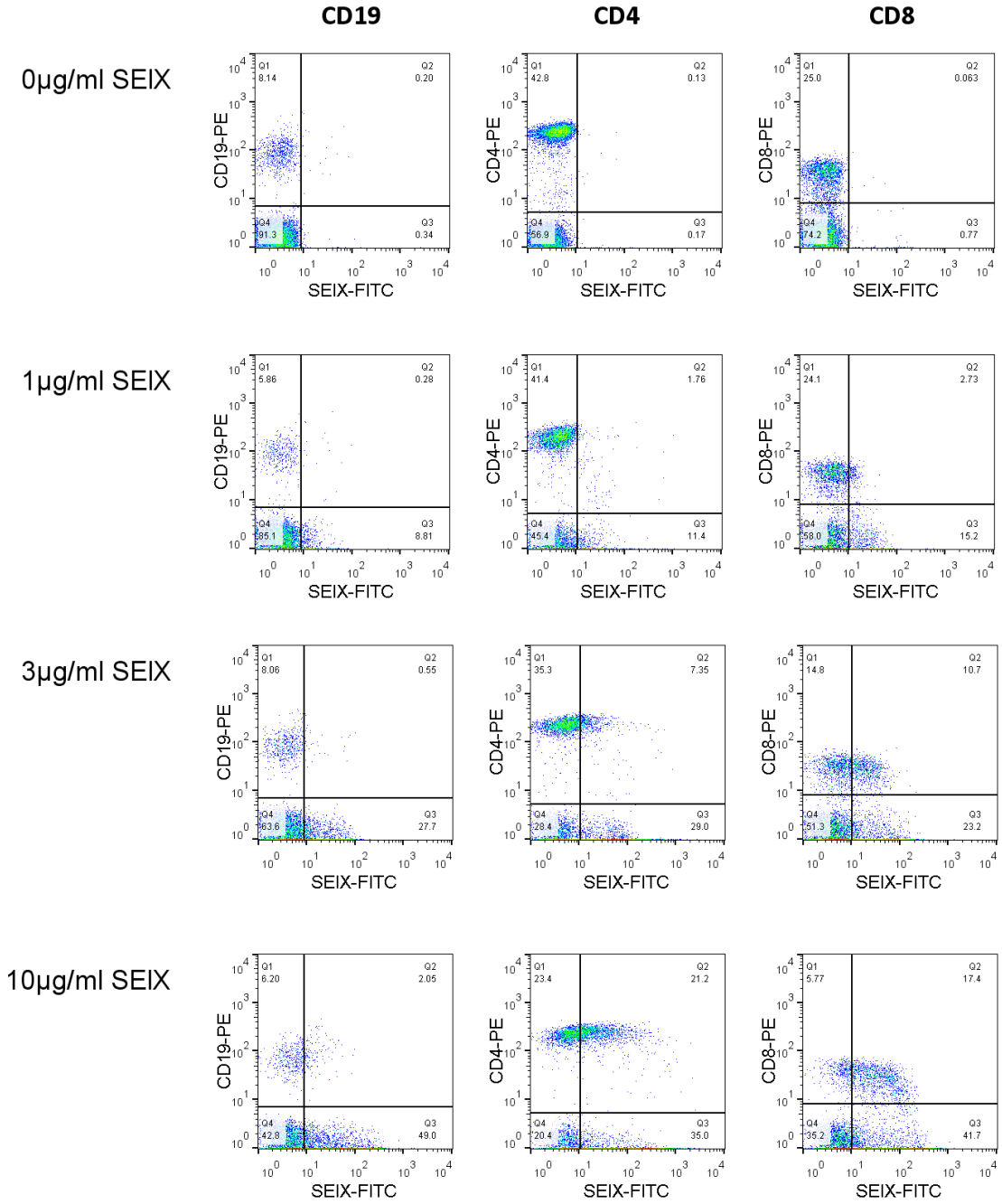


Figure 4.2 SEIX binding to different lymphocyte subpopulations. Two-colour flow cytometry was used to analyse SEIX binding to different lymphocyte subpopulations. To differentiate for specific lymphocyte subpopulations, T-lymphocytes ($CD4^+$ and $CD8^+$), and B-lymphocytes were concurrently stained with PE-conjugated antibodies directed against CD4, CD8, or CD19. SEIX binding was determined using a FITC conjugate mouse anti-HIS-tag antibody.

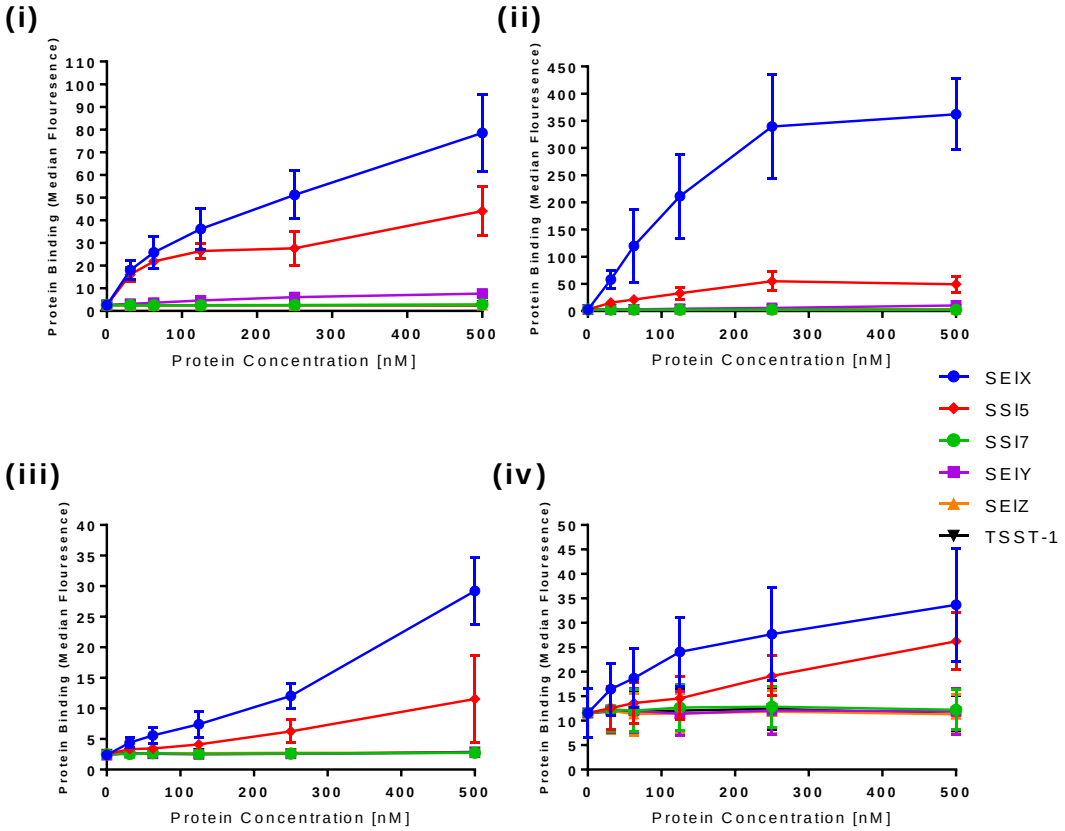
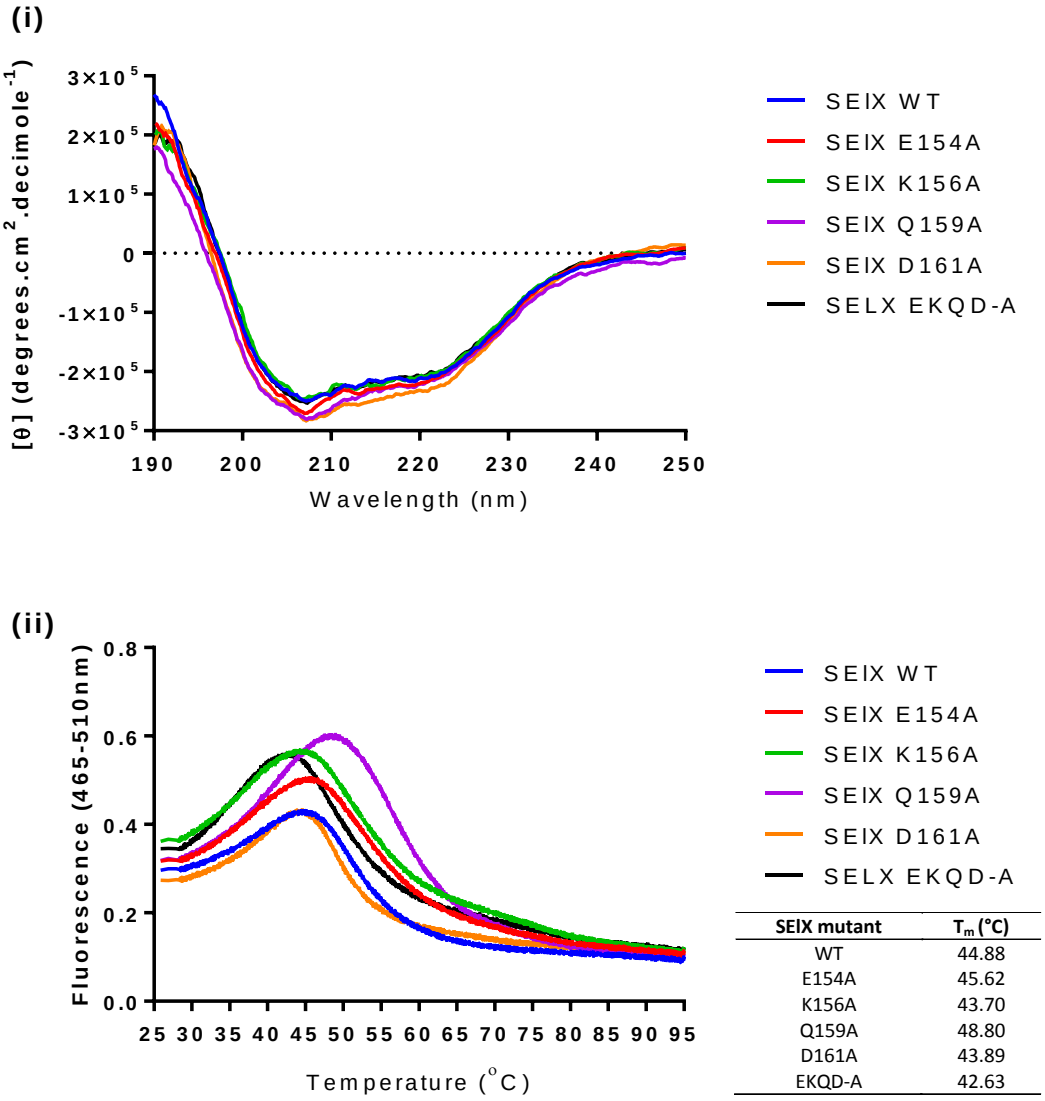


Figure 4.3 SEIX interacts with neutrophils from a range of mammalian species. Flow cytometry analysis of binding of recombinant staphylococcal proteins to isolated human (i), murine (ii), bovine (iii) and laprine (iv) neutrophils. Binding to cells was detected using mouse anti-HIS-FITC IgG binding to the 6 x HIS-tag on the recombinant proteins. Mean median fluorescence of three donors is shown ± standard error of the mean. SSl5 and SSl7 were used as positive and negative controls respectively.

4.3.3 SElX binding to PMNs is dependent on the predicted sialic acid binding motif.

SElX was demonstrated in chapter 3 to contain a sialic acid binding motif which has been reported to mediate the interaction between some SSl-proteins and neutrophils. To test if the SElX interaction with PMNs is sialic acid-dependent, binding assays were performed on neuraminidase-treated cells, as previously reported (Fevre et al., 2014). Neuraminidase treatment of neutrophils prior to incubation with SElX abolished neutrophil binding suggesting that binding involves a sialic acid interaction (Fig 4.5). In order to confirm the role of the predicted sialic acid binding motif in binding to neutrophils, site directed mutagenesis was carried out. Single site directed mutants of each of the four predicted sialic acid binding residues were prepared, in addition to a combination mutant with all four residues replaced with alanine. Mutagenized proteins were expressed and purified under identical conditions and structural validation by circular dichroism and thermal shift was performed to ensure the mutations had not destabilised the proteins (Fig 4.4). The proteins were then examined for leukocyte binding capacity by flow cytometry analysis. The single site directed mutants SElX –E154A, K156A, Q169A and the combination mutant SElX-EKQD-A exhibited almost complete loss of binding to neutrophils and monocytes compared to the WT rSElX suggesting an important role for these residues in neutrophil binding of SElX. The mutant SElX-D161A bound to neutrophils and monocytes at an equivocal level to WT rSElX, suggesting this residue is not essential for neutrophil binding. Taken together these data show that the predicted sialic acid binding motif (SAB) encoded in SElX is required for binding to neutrophils and monocytes.



SEIX mutant	T_m (°C)
WT	44.88
E154A	45.62
K156A	43.70
Q159A	48.80
D161A	43.89
EKQD-A	42.63

Figure 4.4 Mutations in the predicted sialic acid binding site of SEIX have limited impact on protein structure and stability. Circular dichroism analysis was performed on SEIX SAB mutants to ensure the secondary structure was not affected by the mutations (i). Thermal shift assays were performed to check the stability by thermal shift (ii). The T_m of each mutant was determined by calculating the temperature at which the fluorescence peaked.

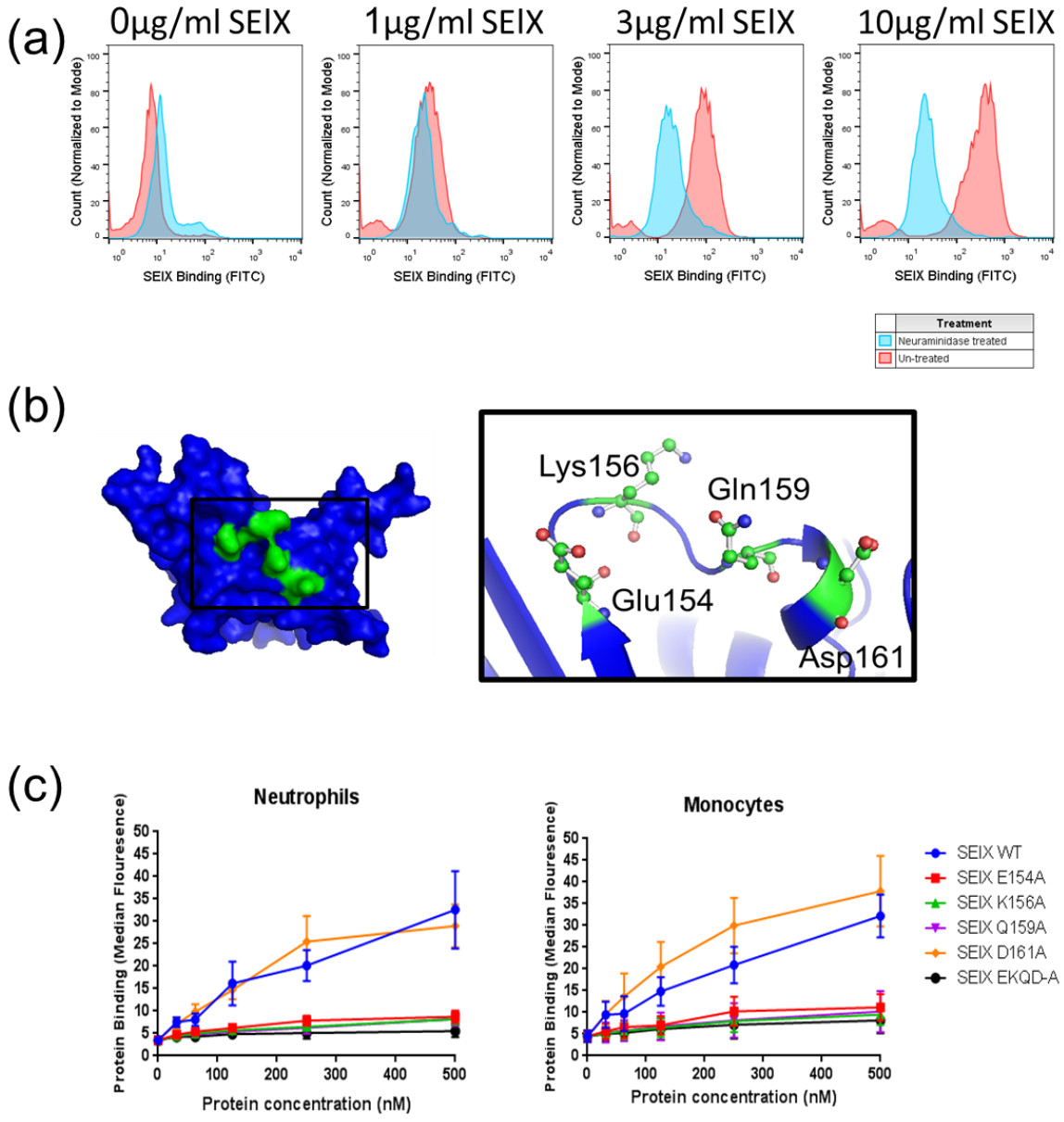


Figure 4.5 SEIX interaction with Neutrophils is mediated via the sialic acid binding motif. (a) Flow cytometry analysis of SEIX binding to human neutrophils treated with 0.2 U/ml neuraminidase versus untreated cells. 6 x His-tagged SEIX binding was detected using Mouse anti-His FITC IgG. (b) Protein model of SEIX showing the location of sialic acid binding residues (coloured green) in the protein structure these residues are magnified in the black box showing their atomic structure in the binding pocket. (c) Flow cytometry assessment of site directed mutants of the sialic acid binding pocket in SEIX. Each of the residues in part (b) has been exchanged for an alanine. Binding to human cells was determined using flow cytometry and binding was detected using Mouse anti-His FITC IgG.

4.3.4 SElX interacts with different neutrophil glycoprotein receptors.

This section of the study was performed by Dr David James and supervised by Prof. Victor J Torres based at New York University Medical Centre. Affinity precipitation analysis with human PMN lysates suggests that SElX can interact with multiple neutrophil proteins. The quantitative mass spectrometry analysis demonstrated that at least 12 proteins were enriched by SElX-coated Ni-NTA beads more than 5 fold higher than uncoated Ni-NTA beads (Table 4.2). The enriched proteins represent a broad range of functional pathways in the neutrophil but the majority of enriched proteins were identified as surface glycoproteins. Of note, the most enriched proteins are four glycoproteins that display integrin or cell activation functions (CD45, CD13, CD31 and CD148). Another protein that was found to be highly enriched was maltase-glucoamylase (MGAM) which is an enzyme found in the gelatinase and ficolin granules of neutrophils (Rorvig et al., 2013). Other cytosol or granule proteins enriched by SElX include p22-PHOX and α-antitrypsin suggesting that the SElX interaction is not limited to neutrophil surface proteins. MGAM, p22-PHOX and α-antitrypsin all are all involved in the microbicide functions of neutrophils. If SElX is internalised then these cytosolic proteins could provide targets for SElX which could disrupt the neutrophil and immune function.

The affinity precipitation experiment was repeated with the SAB mutants of SElX and the reduction in protein enrichment that was observed (Fig 4.6) reflected the decrease observed in the leukocyte binding assay (Fig 4.5). This suggests that many of the proteins enriched during the precipitation assay are dependent on the predicted sialic acid binding motif. The mass spectrometry analysis was repeated (across 2 donors) and the peptide enrichment of CD45, CD31, CD13, CD148 and MGAM by each SAB mutant was determined. MS analysis was only possible on precipitation experiments from 2 donors, therefore no statistical analysis could be carried out. Mutants E154A and K156A and D161A exhibited limited reduction in enrichment for the 5 proteins analysed. However E154A did

bind differentially to CD31 and CD13 suggesting that this residue can contribute to specific binding of these receptors. The mutant Q159A exhibited a diminished enrichment for all 5 proteins analysed suggesting an important role in the interaction between SElX and the sugar molecules on its glycosylated targets. Overall, enrichment by SElX EQKD-A demonstrated the lowest level of enrichment of all the SAB mutants tested. For SElX EKQD-A peptide enrichment was attenuated for all five neutrophil proteins analysed, especially CD31. Taken together these data indicate the interactions between SElX and neutrophil proteins observed are mediated by the predicted sialic acid binding motif.

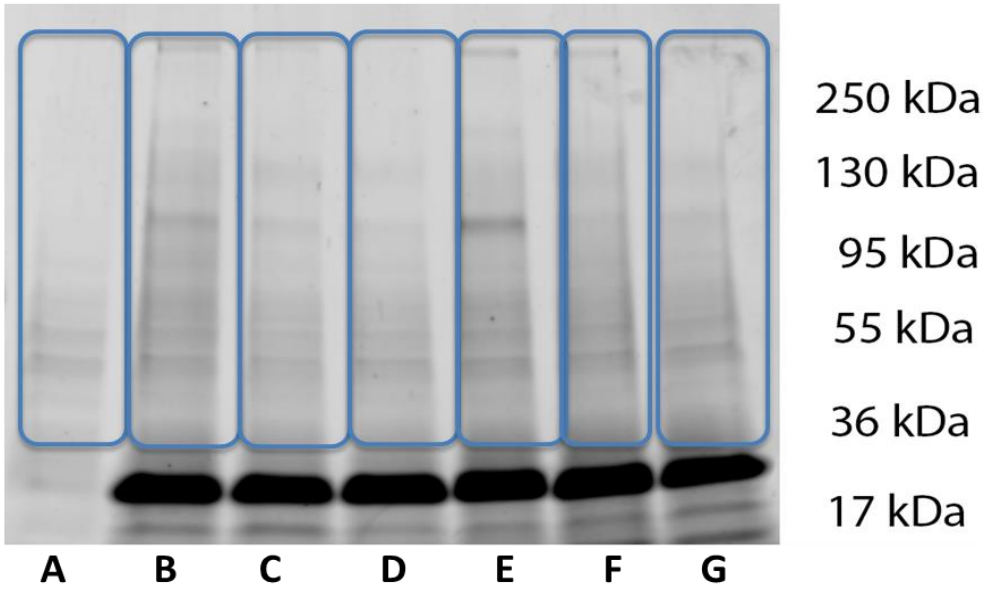
Table 4.2 MS results for proteins enriched by SElX from neutrophil lysates.

Protein	Size (kDa)	Accession	Function[a]	Fold Enrichment vs no protein control
CD45	130.8	P08575-2	Protein tyrosine-protein phosphatase, critical in T-cell activation. Dephosphorylates LYN (Src family of kinases) and modulates LYN.	>10 fold 5/5 donors
CD31	109.5	P16284-3	Expressed on platelets and leukocytes and is primarily concentrated at the borders between endothelial cells. CD31 has been shown to interact with the Src family of kinases (Cicmil et al., 2000).	>10 fold 5/5 donors
CD13	80.2	P15144	Alanine aminopeptidase involved in cell differentiation development and metabolism. Is up regulated during neutrophil apoptosis (Hart et al., 2000).	>10 fold 5/5 donors
CD148	145.9	Q12913	CD148 is a receptor-like protein tyrosine phosphatase up-regulated on T cells after T cell receptor stimulation. It has also been shown to interact with the SRC family of kinases.	>10 fold 3/5 donors 8 fold 2/5 donors
MGAM (maltase-glucoamylase)	311	E7ER45	Enzyme with polysaccharide substrates shown to be a component of neutrophil Gelatinase and Ficolin Granules (Rorvig et al., 2013)	4-10 fold 5/5 donors
CD18	84.7	P05107	Integrin β2 involved in cell signalling and activation. Associated with CD11a, CD11b and CD11c forming critical leukocyte signalling complexes.	>10 fold 2/5 donors 9 fold 1/5 donors
CD11A; LFA-1; LFA1A	119.1	P20701-3	Isoform 3 of Integrin alpha-L involved in cell signalling and activation. Associated with CD18 forming critical leukocyte signalling complexes.	4-10 fold 2/5 donors
CD11b	127.1	P11215	Integrin alpha-M involved in cell signalling and activation. Associated with CD18, forming critical leukocyte signalling complexes.	2-4 fold 2/5 donors

Protein	Size (kDa)	Accession	Function[a]	Fold Enrichment vs no protein control
Desmoplakin	331.6	P15924	Protein critical in the formation of desmosomes.	>10 fold 1/5 donors
p22-PHOX	65.3	P04839	Cytochrome b-345 heavy chain, cytochrome b has been implicated as the primary component for involved in the oxidase system used to destroy microbial components	4-10 fold 2/5 donors
Haptoglobin	45.2	P00738	Haptoglobin captures, and combines with free plasma hemoglobin to allow hepatic recycling of heme iron and to prevent kidney damage.	>10 fold 1/5 donors 2 fold 2/5 donors
CD50	59.5	P32942	Ligand for the β2 integrins	5-9 fold 3/5 donors
Alpha-1-antitrypsin	46.7	P01009	Protects tissue from damage by proteases, especially neutrophil elastase.	5-9 fold 3/5 donors
CD44	37.3	B6EAT9	Hyaluronate receptor that can mediate lymphocyte activation, cell migration and variable glycosylation of this protein can mediate direct binding to fibrin and immobilised fibrinogen.	5-9 fold 2/5 donors
Matrix metallopeptidase 9	66.1	B7Z747	Along with elastase, appears to be a regulatory factor in neutrophil migration across the basement membrane	2-4 fold 2/5 donors
Chloride intracellular channel protein	26.9	O00299	Cell metabolism, internal pH regulation and cell stabilisation	2-4 fold 2/5 donors
CD66c	37.2	P40199	Involved in neutrophil adhesion to fibronectin (Nair and Zingde, 2001)	5-9 fold 3/5 donors

[a] Functional information was obtained from NCBI Entrez entry unless otherwise stated

(i)



A: Non-specific binding (Ni-NTA beads only)
B: WT
C: E154A
D: EKQD-A
E: D161A
F: K156A
G: Q159A

(ii)

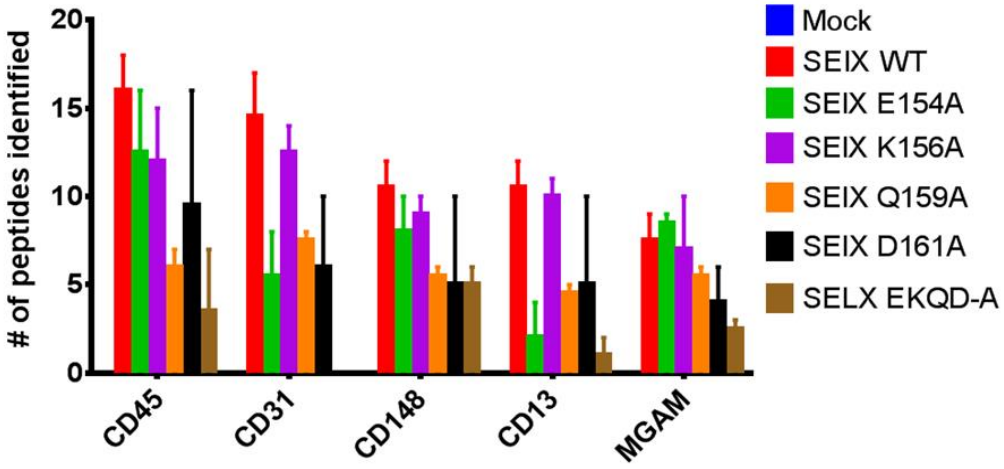


Figure 4.6 SElX binding mutants exhibit differences in protein enrichment for neutrophil lysates. (i) SDS-PAGE analysis of affinity precipitation experiments of SElX and SAB mutants with neutrophil lysates. Gels were stained with sypro ruby. Gel fragments in the blue boxes were excised and submitted for quantitative mass spectrometry analysis and compared to the non-specific binding sample. (ii) Proteins showing the highest level of enrichment from the SElX affinity precipitation with neutrophil lysates were analysed for peptide enrichment following incubation with SAB mutants. Data shown indicates the mean number of peptides detected from each enriched protein (± SEM from two donors). All data was acquired by Dr David James and supervised by Victor J Torres based and NYU medical centre.

4.3.5 SElX interacts with CD50 in a sialic acid dependent manner.

To determine if SElX was interacting directly with any of the protein targets indicated by the affinity precipitation analysis, ELISAs were performed with recombinant glycoprotein receptors. PSGL-1 (CD162) was selected as this had already been shown to interact with SElX (Fevre et al., 2014). Intercellular adhesion molecule 3 (ICAM-3) (CD50) was chosen from the affinity precipitation analysis as this is an important signalling molecule involved in a number of important functions including induction of apoptosis and cell to cell signalling (Costantini et al., 2011, Kessel et al., 2006). ICAM-3 was also one of the few receptors from the precipitation analysis that was available as a recombinant protein and had been prepared in a mammalian expression system. A mammalian expression system is important given the likely role of glycosylation in the binding of SElX to its target. The three proteins examined in this section were obtained from sources that had expressed these proteins in mammalian cells (Life technologies, UK). The FcγR CD16b was selected as a negative control. The proteins were added to plates coated with SElX, SElX EKQD-A, SSl5 and SSl7. SSl5 showed a high affinity for all three proteins tested suggesting that SSl5 can bind to a range of glycoproteins (Fig 4.7). As expected, SSl7did not interact with any of the glycoproteins tested. SElX demonstrated a strong interaction with PSGL-1 as previously shown (Fevre et al., 2014). Of note, SElX EKQD-A bound to PSGL-1 at a similar level as the WT suggesting this interaction is independent of the predicted sialic acid binding site. In contrast the SAB mutant exhibited a significant reduction in binding to ICAM-3 suggesting an interaction that involves a sialyted receptor.

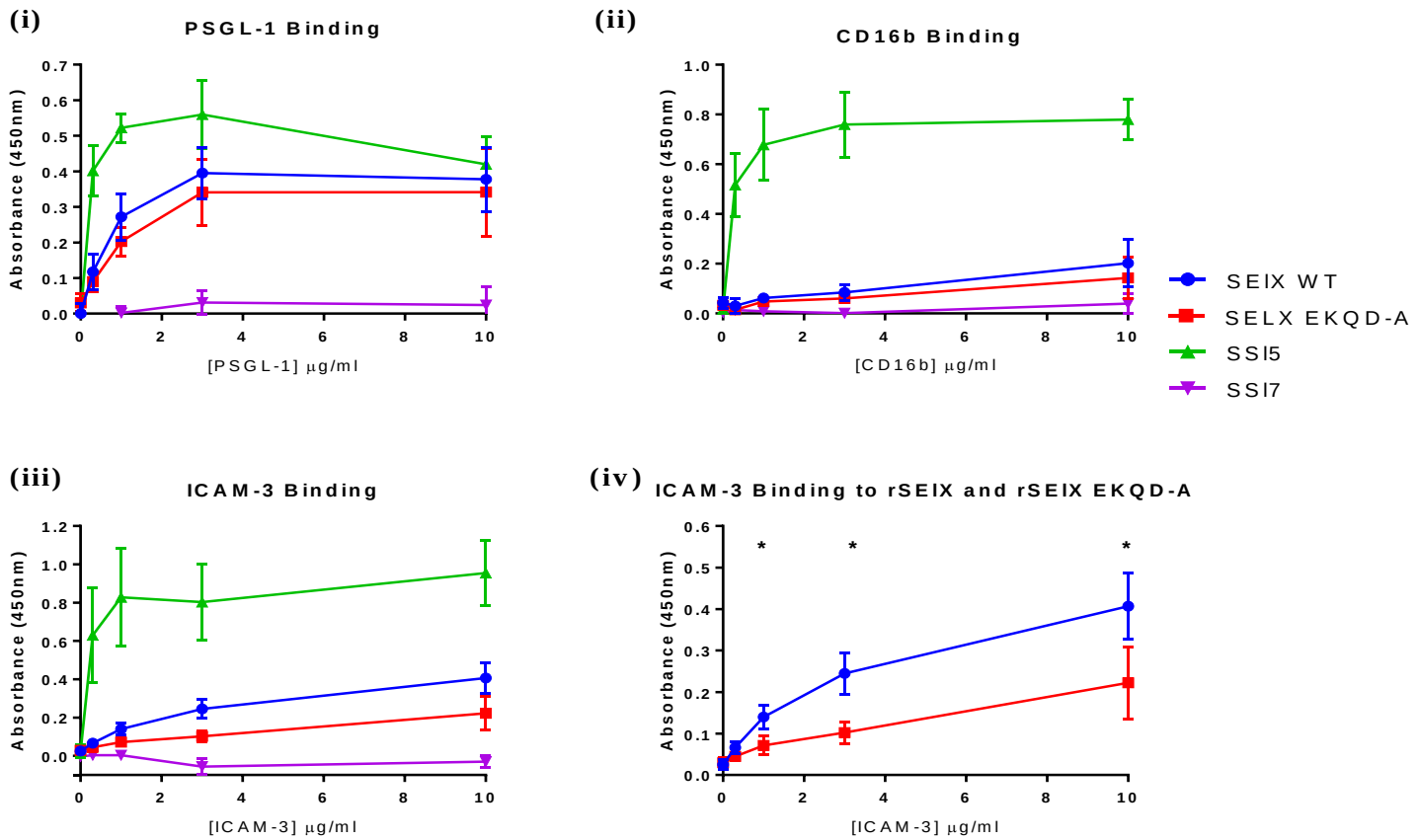


Figure 4.7 SEIX interacts with CD50 in a sialic acid-dependent manner. ELISAs were performed comparing the interaction between staphylococcal proteins and recombinant PSGL-1 (i), CD16b (ii) and ICAM-3 (iii). ICAM-3 binding to SEIX and SEIX EKQD-A was tested by T-test, * denotes a p-vale <0.05.

4.4 DISCUSSION

Nucleotide and derived amino acid sequence analysis in Chapter 3 demonstrated that SElX contains a conserved sialic acid binding motif. This is a common feature of the SSl-proteins binding to the surface of neutrophils and was confirmed to mediate the interaction seen between SElX and human neutrophils and monocytes. When the predicted sialic acid binding motif was attenuated the ability of SElX to bind to neutrophils was diminished, and in the case of the multiple residue mutant completely abolished. The structural analysis of these proteins demonstrated that the mutations had no impact on the folding of the proteins. This demonstrates that the predicted sialic acid binding motif is critical for the interaction between neutrophils and SElX. SElX demonstrates a high affinity for human neutrophils and monocytes, similar to the level seen for that of SSl5. The interaction with neutrophils is replicated in other mammalian species and is suggestive of an important role for SElX in *S. aureus* pathogenesis in mammalian hosts.

SElX exhibited strong binding to human neutrophils and monocytes as previously shown (Fevre et al., 2014). In this work we have also demonstrated that there was some binding to human lymphocytes but this was limited to $CD4^+$ and $CD8^+$ T-cells and there was no binding to B-lymphocytes. This is a characteristic of the SSl-proteins that bind to human leukocytes and may reflect the low activity of glycol-transfereases in peripheral lymphocytes prior to activation (Kumar et al., 1998). This would suggest that if lymphocytes become activated during infection then SElX or SSl-proteins may be able to target them in addition to neutrophils and monocytes. In lymphocytes, glycol-transfereases are activated by the presence of IL-2 which is an interleukin that can be induced by SAg-mediated activation of the lymphocyte (Kumar et al., 1998). It is possible that SElX can induce glycoprotein activation on the surface of lymphocytes then act as a glycoprotein antagonist modulating lymphocyte function.

SElX demonstrates the ability to interact with neutrophils from a range of mammalian species. The interaction with murine cells is noteworthy as it allows SElX to be potentially studied in a model where there is no interference from the superantigenicity of the protein. Classically, SAgs have not been studied in mouse models due to the differences in MHC class II alleles which prevent SAgs from activating murine lymphocytes. Although, this has not been demonstrated yet for SElX, lack of mitogenicity in mice is likely given its relationship to SAgs like TSST-1 (Tilahun et al., 2011). SElX demonstrated the highest level of affinity for murine neutrophils, which may reflect the source of the cells. Unlike the other mammalian species tested murine neutrophils were isolated from bone marrow and not peripheral blood. It is possible that these cells display a different amount of glycoprotein receptors to peripheral cells. Rabbits are also another important model for *S. aureus* infections and the ability of SElX to bind to neutrophils from this species suggests that the role of neutrophil binding of SElX in *S. aureus* pathogenesis can be examined in this model. The mammalian species, rabbits and cattle, represent important veterinary hosts of *S. aureus* (Fitzgerald, 2012). The ability of SElX to bind to neutrophils from these species suggests this function is retained in different host species, suggesting a broad role for SElX across different host species. Importantly, the SAB identified in Chapter 3 was demonstrated to be highly conserved among alleles of SElX from human and animal strains (Fig 3.7).

The other SAgs analysed in this study did not have the same level of binding affinity for neutrophils and monocytes as SElX with both TSST-1 and SElZ not exhibiting any binding to leukocytes. However SElY had a relatively low affinity interaction with neutrophils and to slightly greater extent monocytes in spite of the lack of a SAB. These data suggest that SAgs in addition to SElX have the capacity to bind to leukocytes via multiple mechanisms.

The affinity precipitation analysis demonstrated that SElX can interact with an array of glycoprotein receptors, the most enriched were CD45, CD13, CD31 and CD148 and have

been shown to be involved in integrin function and cell signalling (Liu et al., 2012, Hart et al., 2000, Hermiston et al., 2009). CD45 and CD148 are both involved in activation of Src family kinases and cell activation (Hermiston et al., 2009), whereas CD31 is present on leukocytes and platelets and has multiple functions including cellular adhesion and activation (Liu et al., 2012). CD31 has also been shown to be associated with the Src family of kinases which upon activation leads to CD31 phosphorylation increasing adhesion of the protein to endothelial surfaces (Cicmil et al., 2000, Liu et al., 2012). These data suggests that SElX may bind to receptors involved in neutrophil activation and transmigration.

In addition, SElX was also shown to interact with a number of intracellular proteins including MGAM, p22-PHOX and α-1-antitrypsin. This suggests that SElX may be internalised or expressed intracellularly allowing the targeting of intracellular pathways of neutrophil function. This has precedent as other several SSl-proteins are rapidly internalised by neutrophils and have been demonstrated to inhibit function (Baker et al., 2007, Chung et al., 2007, Hermans et al., 2012).

When the affinity precipitation experiments were repeated with the SAB SElX mutants the enrichment of proteins from neutrophil lysates was reduced. For the mutants K156A and D161A enrichment was unaffected for all five of the proteins analysed. This suggests that these residues are not essential in coordinating the interaction between SElX and these protein targets. The SAB mutant Q159A showed a reduced enrichment for all five neutrophil proteins suggesting the importance of this residue in the neutrophil interaction. The mutant E154A showed differential enrichment to the glycoprotein targets with 2 of 5 proteins diminished, suggesting this residue may confer a level of specificity to the SAB. The EKQD-A mutant of SElX exhibited reduced enrichment to all five protein binding partners. Taken together these data demonstrate the predicted SAB is required for SElX to interact with the binding partners identified by affinity precipitation analysis and that all four amino acid residues examined contribute to the binding of target proteins.

PSGL-1 was shown to interact with SElX by ELISA and in the current study ICAM-3 was also shown to interact with SElX. Unlike PSGL-1, there was a significant reduction in binding of ICAM-3 to SElX EKQD-A, consistent with a sialic acid binding activity that mirrors the binding interaction seen on the surface of neutrophils. Of note ICAM-3 is a ligand for important activator pathways such as MAC-1 and LFA-1 (de Fougerolles and Springer, 1992, Petruzzelli et al., 1995). Blocking ICAM-3 can interfere with the activation of NK-cells and lymphocytes when these cells co-localise (Costantini et al., 2011, Petruzzelli et al., 1995). It has also been shown that ICAM-3 ligation in neutrophils can induce apoptosis and it is feasible that SElX may be able to activate this pathway (Kessel et al., 2006). It is unclear why both the wild type SElX and the SAB mutant bound to PSGL-1 at a similar level, but we speculate that SElX has an alternative binding site for PSGL-1.

In the current study we have demonstrated that SElX can bind to neutrophils and monocytes through a predicted SAB which is found in other SSl-proteins. Affinity precipitation analysis has suggested SElX can interact with a range of proteins from neutrophils which are critical to immune function. The functional impact of SElX is yet to be determined and will be examined in detail in the next chapter along with SElY.

Chapter 5

Impact of SEIX on Neutrophil Function

5.1 INTRODUCTION

S. aureus has been demonstrated to produce an array of factors that interact with PMNs modifying their function through multiple mechanisms including direct toxicity or inhibition. Of note, the SSl-proteins can interfere with PMN immune pathway functions through numerous ways. For example, SSl5 interferes with integrin function (Bestebroer et al., 2007), and in the case of SSl3 binding of the protein inhibits cell activation by blocking of TLR2 (Bardoel et al., 2012). Other SSl-proteins have been shown to interact with parts of the humoral immune system. For example, SSl7 can bind to both C5 and IgA and interfere with the complement pathway assisting *S. aureus* immune evasion (Bestebroer et al., 2010, Langley et al., 2005, Lorenz et al., 2013). SSl10 is another example of a complement inhibitor which binds to the IgG Fc region, blocking the activation of the classical complement pathway (Itoh et al., 2010b, Patel et al., 2010). SSl8 has been demonstrated to interact with tenascin C and to interfere with wound healing by retarding the motility of keratinocytes (Itoh et al., 2013).

We have shown that SElX binds to PMNs through the predicted glycan binding motif found in some SSl-proteins (Hermans et al., 2012). For SElX the functional consequences of this interaction are unknown but it is reasonable to speculate that it influences neutrophil function. SElX has already been demonstrated to act as a superantigen, but to date no other SAg has been reported to bind to leukocytes independently of the T-cell activation pathway. As such the molecular mechanisms underpinning the distinct function of SElX remain to be elucidated. A number of *S. aureus* virulence factors have been demonstrated to exhibit two or more functions. For examples, the collagen binding adhesin (Cna) has been demonstrated to bind to C1q (in addition to collagen) and prevent activation of the alternative complement pathway (Kang et al., 2013). In addition, FLIPr binds the formylated peptide receptor and

can induce inflammation, block the Fcγ-receptors and inhibit IgG-mediated phagocytosis (Prat et al., 2006, Stemerding et al., 2013).

The aim of this part of the study is to determine if SElX has any functional impact on neutrophils and if this activity is distinct from its T-cell mitogenicity. The objectives include:

- To test if the identified SElX neutrophil binding activity is distinct from its superantigenic activity.
- Determining if SElX can enhance bacterial survival when incubated with neutrophils.
- Analyse the functional impacts that SElX binding has on neutrophils.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of human serum and antibodies

Blood donations were obtained from 10 healthy volunteers (5 male and 5 female) according to the protocol outlined in section 2.1. Blood was sampled without anticoagulant and following sampling was transferred into 50 ml conical tubes (BD falcon). Blood was incubated overnight at 4°C and allowed to clot. Following the overnight incubation the samples were centrifuged at 2000 x g for 10 min and serum supernatant was harvested and pooled with sera from the other donors. Pooled serum was filtered through a 0.45 μm membrane (Merk Millipore, Germany) to remove precipitates before snap-freezing on dry ice, aliquoted and stored at -80°C. When required, aliquots were defrosted on ice and for heat-inactivated serum an incubation step of 56°C for 30 min was employed. Purification of whole human IgG was performed using a Hitrap Protein G HP column (GE healthcare) on an AKTA FPLC machine (GE healthcare, Buckinghamshire, UK).The column was installed and run according to the manufacturer's specification. IgG was purified by diluting the serum 1/10 in PBS before being bound to the column. Following binding IgG was washed with PBS and eluted with 100 mM glycine (pH 2.7) buffer. The eluted antibodies were immediately dialysed into pH 7.3 PBS using Float-a-lyser units (10,000 MWCO) (Spectrum Laboratories, CA, USA). Antibody purity was examined by SDS-PAGE analysis and quantified using a Nanodrop spectrophotometer (ND1000) (Thermo Scientific, USA) before storing at -20°C.

5.2.2 Bacterial survival assays

Bacteria were cultured to an OD_{600} of 4.0 in BHI broth and then washed by diluting in PBS followed by centrifugation at 4000 x g for 10 min. The cells were then diluted to $5x10^4$

cfu/ml in HBSS containing 10 % (v/v) complement-inactivated serum. Isolated human neutrophils were added at a ratio of 1 bacterium to 10 PMNs, and incubated for either 15 or 30 min at 37°C with shaking. Recombinant SElX was added to neutrophils to a final concentration of 10 μg/ml and incubated at room temperature for 30 min before the addition of the bacteria. 100 μl volume of the neutrophil bacterial suspension was diluted into 900 μl of PBS containing 0.05 % (v/v) Triton X 100 and incubated for 5 min at room temperature to lyse the neutrophils. Viable bacteria in each reaction mixture were counted following a 10-fold dilution series in PBS. 100 μl of each sample was spread onto TSA plates, followed by overnight incubation at 37°C, surviving bacteria were enumerated and compared to the initial bacterial inoculum to determine percentage bacterial survival.

5.2.3 Phagocytosis assays

Phagocytosis was measured with FITC-labelled *S. aureus*. Briefly, CA-MRSA USA300 *spa*::Tn (Table 5.1) was grown overnight and diluted to an OD_{600} of 0.5 followed by washing in PBS and centrifugation at 4000 x g for 10 min. The bacteria were re-suspended in an equal volume of PBS with FITC in DMSO to a final concentration of 0.5 mg/ml, and incubated in the dark at 4°C for 30 min. The bacteria were washed with PBS (4000 x g for 10 min) to remove the excess FITC before the cells were re-suspended in assay media to an OD_{600} of 0.5. In 2 ml 96-well v-bottomed plates (Corning, USA), FITC labelled *S. aureus* were mixed with human serum, complement-inactivated serum or purified IgG for 15 min at 37°C to facilitate opsonisation. Subsequently, isolated human neutrophils, with or without inhibitor (pre-incubated for 30 min at room temperature), were added at a 10:1 bacterium/cell ratio and incubated for 15 min at 37°C with shaking at 750 rpm. The reaction was stopped with 1 % (v/v) paraformaldehyde (Fisher Scientific, UK), and cell-associated fluorescent bacteria were analysed by flow cytometry. Phagocytosis was defined as the

percentage of cells with a positive fluorescent signal. Reduction in phagocytosis was calculated by normalising the percentage from test samples to that of uninhibited cells for each opsonin.

Table 5.1 *S. aureus* strains used in this study.

Strain	Description	Reference
USA300 LAC	Wild Type	(Voyich et al., 2006)
USA300 LAC∆*selx*	Chromosomal deletion of *selx*	(Wilson et al., 2011)
USA300 LAC∆*selx* rep	Chromosomal repair of *selx*	(Wilson et al., 2011)
USA300 *spa*::Tn	Transposon insertion into *spa*	(Fey et al., 2013)

5.2.4 Cell line binding experiments

For binding of recombinant SElX to Fcγ receptors a transfected human B-cell lymphoma line (5×10^6 cells/ml) was incubated with increasing concentrations of 6 x HIS-tagged recombinant protein in RPMI 1640 (Gibco, UK) supplemented with 0.05 % HSA (Sigma Aldrich, Dorset, UK) for 30 min on ice. After washing binding was detected using a FITC-labelled monoclonal mouse anti-HIS-tag monoclonal antibody (LS Bioscience, WA, USA). Following washing the cell line population was determined based on forward and sideway scatter on a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lanes, NJ, USA) and fluorescence measured.

5.2.5 Cytotoxicity determined by lactate dehydrogenase (LDH) release

Human PMNs were isolated and re-suspended in HBSS (Gibco, UK) at a concentration of $1x10^5$ cells/ml, followed by the addition of recombinant *S. aureus* proteins and incubated for 1 h at 37°C with 5 % CO_2. Following intoxication, cells were pelleted by centrifugation at 450 x g at 4°C for 5 min. Lactate dehydrogenase (LDH) release was assayed as a measure of PMN viability using the CytoTox-ONE homogeneous membrane integrity assay (Promega, WI, USA) according to the manufacturer's specifications. Briefly, 50 µl of culture supernatant was removed and added to wells containing 50 µl of LDH reagent and incubated for an additional 10 min at room temperature. Fluorescence was measured using a PerkinElmer Envision 2103 multilabel reader (excitation, 555 nm; emission, 590 nm) (PerkinElmer, MA, USA), and data were normalized to 100 % lysis as determined by the addition of 0.2 % Trition X (v/v) to the PMNs. These experiments were performed by Dr David James and supervised by Prof Victor Torres at the NYU Medical Centre.

5.2.6 Determination of apoptosis

For binding of recombinant proteins to leukocytes, neutrophils (5×10^6 cells/mL) and PBMCs (5×10^6 cells/mL) were incubated with increasing concentrations of HIS-tagged recombinant proteins in RPMI 1640 (Gibco) supplemented with 0.05 % HSA (v/v) (Sigma-Aldrich, Dorset, UK) for 30 min at 37°C. After washing with RPMI-HSA, cells were centrifuged at 400 x g for 10 min and then binding of the proteins was detected using a FITC-labelled monoclonal mouse anti-HIS-tag monoclonal antibody (LS Bioscience, WA, USA). The cells were washed one final time and re-suspended in assay media containing the nuclear dye DRAQ 5 (Biostatus, UK). Leukocyte populations were determined based on forward and sideway scatter on a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lanes, NJ) and fluorescence measured. Binding of the protein was determined on the FL1-H channel of this cytometer. DRAQ-5 fluorescence was determined on the FL3-H channel and a higher signal was indicative of nuclear fragmentation, a characteristic of apoptosis.

5.3 RESULTS

5.3.1 The mechanism of neutrophil binding of SElX is distinct from its superantigenicity.

To determine if the superantigenic property of SElX was distinct from its neutrophil binding function, SAB mutants of SElX were examined for their ability to induce T-cell proliferation by [^{3}H] thymidine incorporation. It was found that, despite the manipulation of the sialic acid binding site, all five SAB mutants were capable of inducing T-cell proliferation at comparable levels (Fig 5.1a). Further analysis of the WT recombinant SElX protein and SElX-EKQD demonstrated that overall the superantigenicity of these proteins was not affected (Fig 5.1b), inferring that the residues predicted for sialic acid binding required for binding neutrophils are not required to induce T-cell proliferation.

5.3.2 SElX contributes to enhanced survival of *S. aureus* in the presence of neutrophils.

To investigate the hypothesis that SElX contributes to the resistance of *S. aureus* to neutrophil killing, *S. aureus* strain USA300 LAC and its derivative *selx* deletion mutant (Table 2.1) were incubated with isolated human neutrophils with 10 % (v/v) heat inactivated serum (used as an opsonin to prevent complement mediated killing) (Fig 5.2). After 15 min USA300 LACΔ*selx* demonstrated a trend towards reduced survival but this was not significant (Fig 5.2). At the 30 min time point the survival of USA300 LACΔ*selx* was reduced by over 50% compared to both the WT LAC strain and LACΔ*selx* rep. Re-introduction of *selx* or addition of recombinant SElX restored the survival of LACΔ*selx* to WT levels. Taken together these data indicate that SElX enhances the capacity of *S. aureus* to survive neutrophil mediated killing.

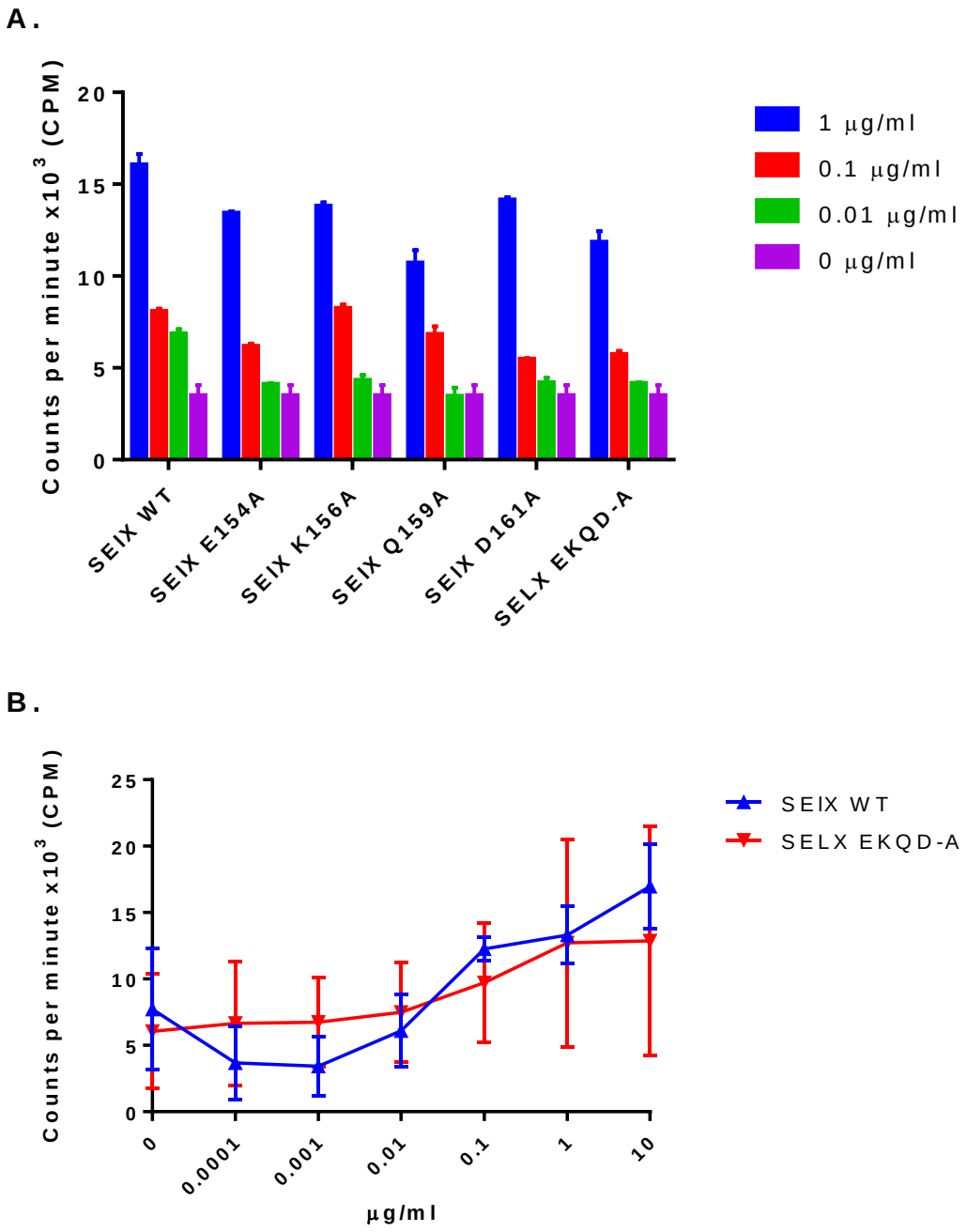


Figure 5.1 Neutrophil binding-deficient mutants of SEIX retain mitogenicity. (A) Isolated human PBMC were stimulated with recombinant WT SEIX and SAB mutants and after 72 h exposure proliferation was determined, as indicated by the incorporation of [^{3}H] thymidine. An experiment analysing the comparative potency of the WT SEIX and mutant SEIX-EKQD was also performed to determine the proliferation induced between 10 µg/ml and 100 pg/ml (B). Results shown are the means of triplicate measurement from 3 human donors ± standard deviation of the mean.

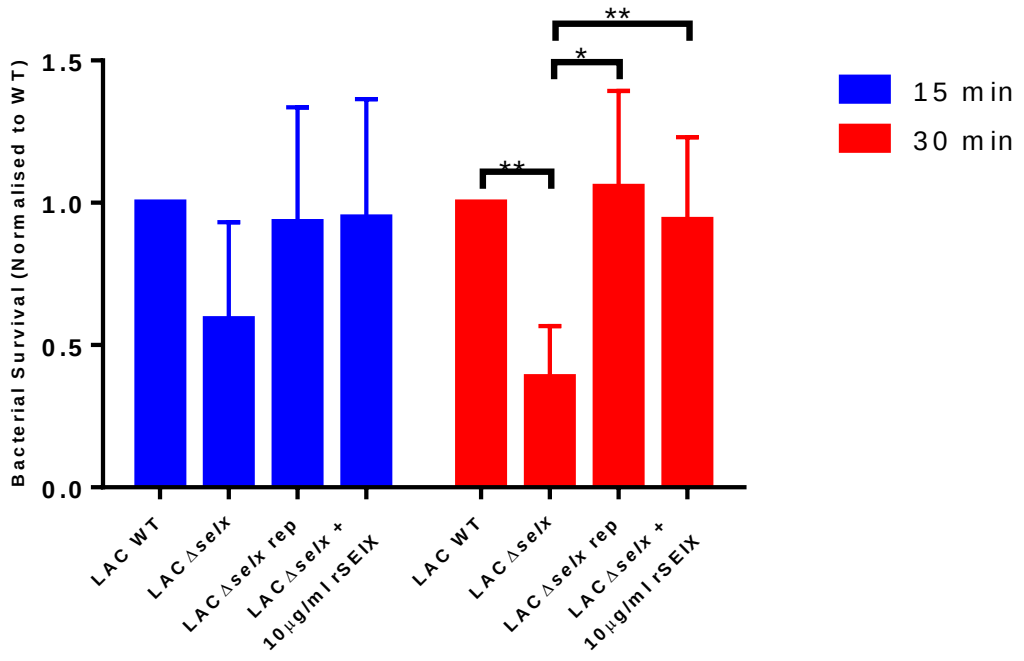


Figure 5.2 SElX enhances *S. aureus* survival in the presence of human neutrophils. *S. aureus* survival assay with human neutrophils. *S. aureus* USA300 and *selx* deletion mutants were grown overnight and then diluted and incubated with isolated human neutrophils for 15 or 30 minutes. rSElX was added to the neutrophils and pre-incubated at room temperature for 30 min. Following incubation neutrophils were lysed with Triton X 100 and surviving bacteria plated and enumerated. Percentage survival was calculated from initial inoculum and the normalised to the wild type. Results shown are the means of 3 independent experiments. Results were tested by T-test, * indicates p value < 0.05 and ** indicates a p value < 0.01.

5.3.3 SElX inhibits IgG-mediated phagocytosis.

To determine if SElX or SElY had any impact on phagocytosis by PMNs, neutrophils where pre-incubated with SElX or SElY before opsonisation and the addition of bacteria. SElX reduced the ability of PMNs to take up bacteria when IgG was used alone as an opsonin (Fig 5.3) and when 1 % heat-inactivated human serum was employed. These data demonstrate that IgG-mediated phagocytosis is inhibited by SElX. In contrast no reduction was seen for full human pooled serum (HPS) suggesting this effect is independent of complement-mediated phagocytosis (Fig 5.3). SElY had no impact on phagocytosis in this assay (Fig 5.3).

Further analysis of SElX-mediated inhibition demonstrated that the activity is highly potent inhibiting IgG mediated phagocytosis at a concentration of 0.4 nM (Fig 5.3). Inhibition of phagocytosis varies from 20 % to 30 % for SElX in comparison to the Fcγ antagonist FLIPr which can reduce the phagocytosis by up 75 %. Of note, no reduction in phagocytosis was observed when the SAB mutant, SElX EKQD-A, was used consistent with a requirement for neutrophil binding dependent on the sialic acid binding site (Fig 5.3).

In order to test the hypothesis that SElX exerts its activity via binding to the Fcγ receptors, the interaction of SElX with the Fcγ receptors was tested. Interaction between SElX and the recombinant Fcγ receptors CD16b was examined in chapter 4 by ELISA and binding was not observed (Fig 4.6). Further, binding was not observed when Fcγ receptors were expressed in a human B-cell lymphoma line (Fig 5.4). These data suggest that the mechanism of inhibition of phagocytosis by SElX is independent of Fcγ receptor antagonism and that IgG-Fcγ receptor signalling is not affected by the SElX interaction with neutrophils.

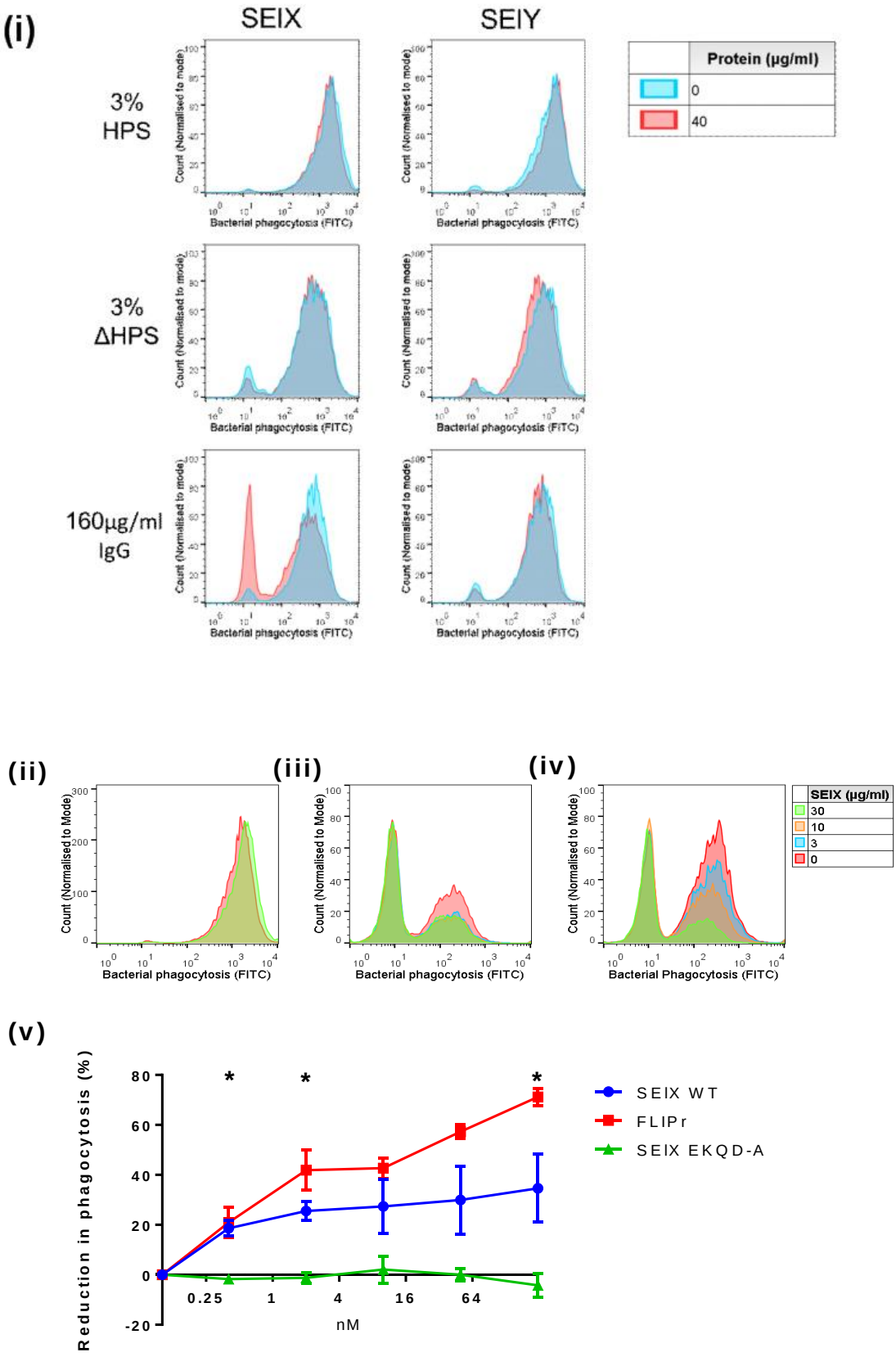

(i)
SEIX
SEIY
3% HPS
3% ΔHPS
160µg/ml IgG
Count (Normalised to mode)
Bacterial phagocytosis (FITC)
Protein (µg/ml)
0
40
(ii)
(iii)
(iv)
Bacterial Phagocytosis (FITC)
SEIX (µg/ml)
30
10
3
0
(v)
Reduction in phagocytosis (%)
nM
0.25
1
4
16
64
SEIX WT
FLIPr
SEIX EKQD-A

Figure 5.3 SElX inhibits IgG-mediated neutrophil phagocytosis. (i) Phagocytosis of fluorescent *S. aureus* opsonized with 3 % (v/v) full human pooled serum (HPS), 3 % (v/v) Heat inactivated human pooled serum (ΔHPS) and purified IgG. Phagocytosis was performed with or without either 40 µg/ml of SElX or SElY. Assay was repeated with 1 % (v/v) full human serum (ii) 1 % (v/v) complement-inactivated serum (iii), or 150 µg/ml of purified IgG (iv) in the presence of SElX at various concentration. Inhibition by SElX was compared to the IgG-mediated phagocytosis inhibitor FLIPr and the SAB deficient mutant SElX EKQD-A (v). Phagocytosis was calculated as the percentage of cells with fluorescent bacteria and expressed relative to buffer-treated cells with 75 µg/ml human IgG. Results shown are the means of three different human donors (error bars SE of mean). Results between SElX WT and SElX EKQD-A were tested by T-test and * indicates a p-value < 0.05.

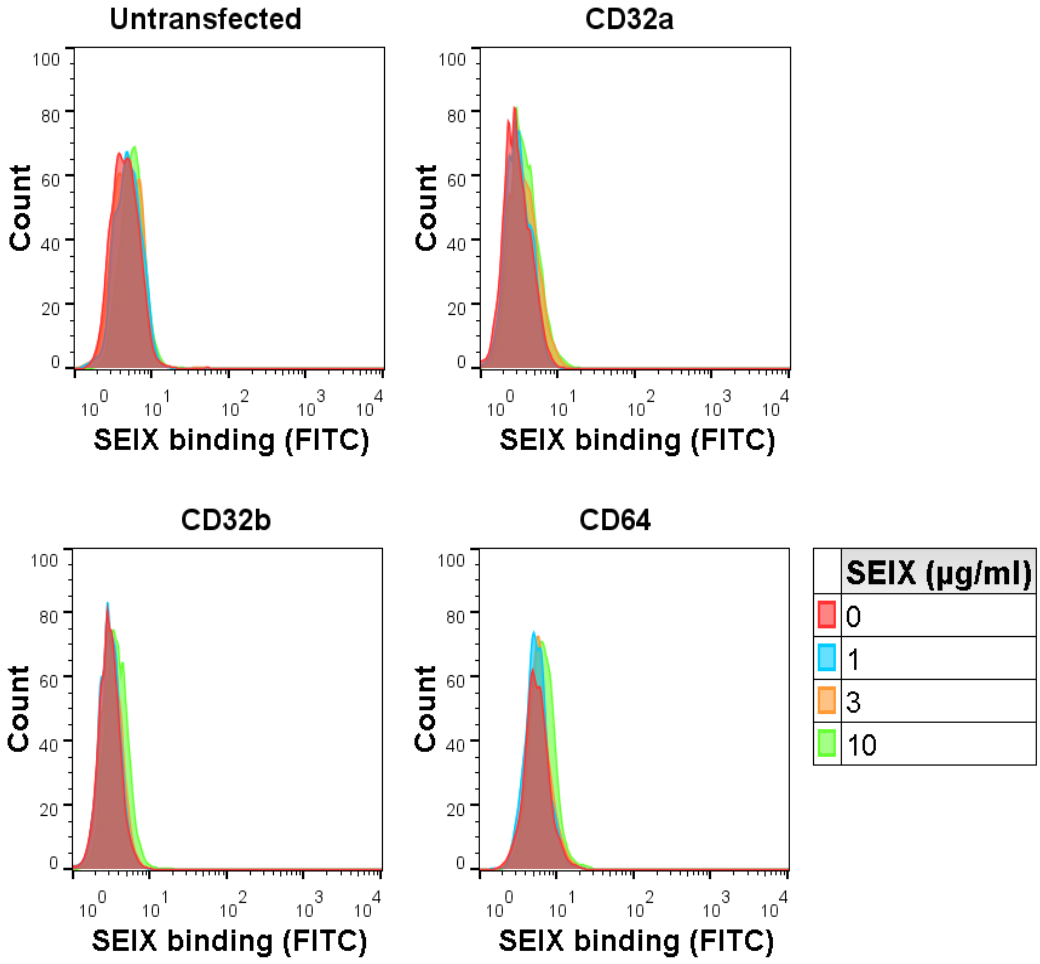


Figure 5.4 SEIX inhibition of IgG mediated phagocytosis is independent of Fcγ receptors. B-lymphoma cells were transfected with Fcγ receptor constructs and SEIX binding was assessed at different concentration. Binding to cells was detected using Mouse anti-His FITC IgG binding to the HIS-tag on the recombinant protein. Data shown is representative of two experiments.

5.3.4 SElX inhibition of phagocytosis is not associated with leukocyte cell death.

We speculated that the mechanism of neutrophil inhibition may be due to cell death induced by SElX. Accordingly, cell death induced via the necrotic and apoptotic pathway was examined. The capacity of SElX to induce LDH release from human PMNs was compared to the leucocidin LukAB (Fig 5.5). As expected LukAB was able to induce necrosis of PMNs down to a concentration below 40 ng/ml. In contrast LDH was not released in response to the high concentrations of SElX tested, suggesting SElX does not induce neutrophil necrosis. These experiments were performed by Dr David James and supervised by Prof Victor Torres at the NYU Medical Centre.

To assess if SElX was capable of inducing apoptosis, the SElX leukocyte-binding assay was repeated and apoptosis was examined by nuclear fragmentation as detected by the nuclear stain DRAQ5. The addition of SElX to human leukocytes had no impact on the level of nuclear fragmentation observed in neutrophils, monocytes or lymphocytes (Fig 5.6). A degree of nuclear fragmentation was observed for neutrophils but this did not increase when SElX was added. For monocytes and lymphocytes nuclear fragmentation was not observed in the presence of or absence of SElX. Taken together these data suggest that SElX does not induce cell death when incubated with human leukocytes and inhibition of phagocytosis is not due to the induction of apoptosis or necrosis.

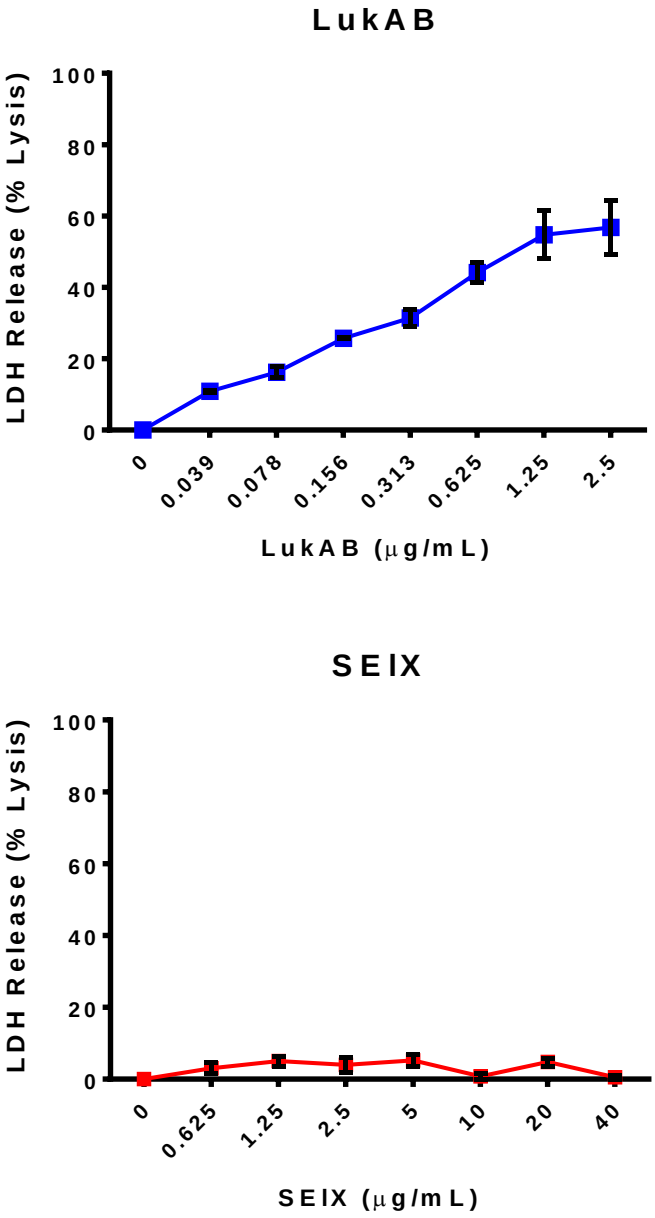


Figure 5.5 SElX does not induce necrosis of human PMNs. Isolated human neutrophils were intoxicated by various concentration of SElX and LukAB. After 1 h incubation the culture supernatants were assayed for the presence of LDH as a proxy for cell lysis. Percentage lysis was determined relative to complete lysis of the neutrophils which was determined by the addition of 0.2 % (v/v) Trition X to the cells. Results shown are the means of three different human donors, ± SD of the mean. These experiments were performed by Dr David James, supervised by Prof Victor Torres at the NYU Medical Centre.

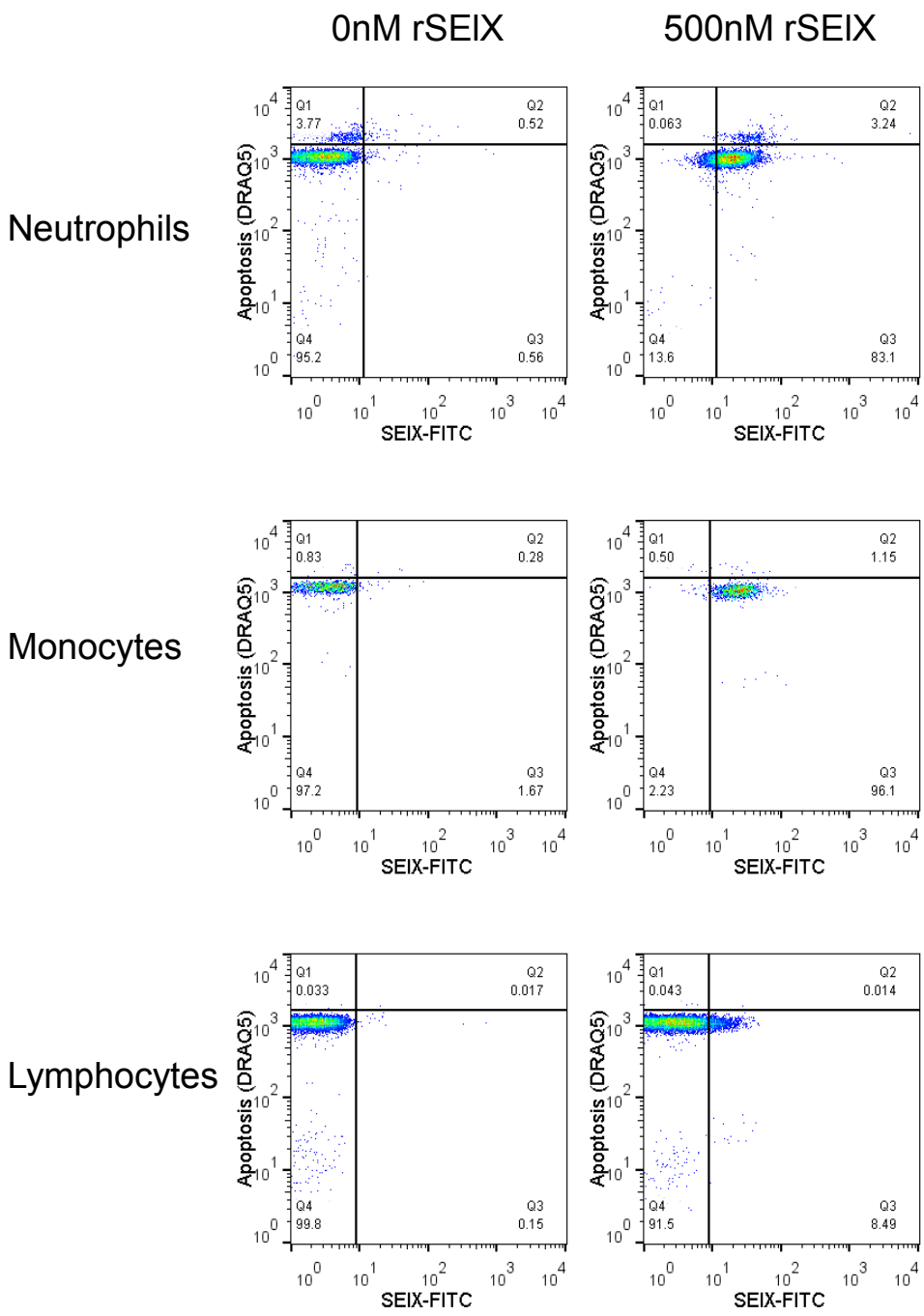


Figure 5.6 SEIX does not induce leukocyte apoptosis. Two colour flow cytometry was for leukocyte binding assays of SEIX and human leukocytes. At the end of the assay the nuclear stain DRAQ5 was added to determine the level of nuclear fragmentation indicating apoptosis. Quadrat plots show the binding of SEIX along the X-axis and the Y-axis shows nuclear fragmentation indicated by higher DRAQ5 fluorescence. These data are representative of three experiments.

5.4 DISCUSSION

Here we have investigated the interaction between SElX and human neutrophils using an array of functional assays. We have demonstrated that SElX has a clear functional impact on the human neutrophil that may enhance immune evasion during *S. aureus* infection. This is the first time a distinct function in innate immune evasion has been demonstrated for a bacterial SAg.

Deletion of the *selx* gene resulted in reduced survival in the presence of neutrophils. Washing of *S. aureus* cells prior to neutrophil incubation should eliminate SElX secreted during culture, implying either that SElX is produced rapidly in response to neutrophil signals before being phagocytosed or alternatively the SElX is produced in the phagosome and enhances bacterial survival after phagocytosis. Of note, the addition of recombinant SElX enhanced the survival of LACΔ*selx*, suggesting a role prior to uptake. Further work needs to be performed to establish if *selx* is being expressed upon uptake of the bacterial cell by the neutrophil and if SElX is acting within the neutrophil to contribute to *S. aureus* survival.

SElX was demonstrated to reduce IgG-mediated phagocytosis by neutrophils independent of the Fcγ receptors. This inhibition was at a lower level compared to the characterised Fcγ receptor antagonist FLIPr, but inhibition by SElX was observed at a concentration as low as 0.4 nM consistent with a relatively high potency that could be physiologically relevant during *S. aureus* pathogenesis. The inability of the SAB mutant of SElX to inhibit phagocytosis demonstrates that binding to the neutrophil surface is essential for functionality which is dependent on the predicted sialic acid binding pocket of the protein. In the section 4.3.4 we demonstrated that SElX could potentially interact with a number of important signalling molecules such as CD50 and components of the MAC-1 signalling complex. It is possible that SElX may bind via these glycoproteins and antagonise

signalling function leading to improper cell activation. Alternatively, internalisation of secreted SElX by the neutrophil may lead to an intracellular effect on neutrophil function. This property has been observed in a number of SSl proteins including SSl4, 5 and 11, although the functional impact of this internalisation is yet to be characterised (Baker et al., 2007, Chung et al., 2007, Hermans et al., 2012). Furthermore some SAgs such as TSST-1 have been shown to be able to mediate their own uptake into cells using the outside-in signalling motif described in chapter 3 (Brosnahan and Schlievert, 2011, Spaulding et al., 2013, Stach et al., 2014).

SElX does not appear to induce cell death as there was no evidence of apoptotic or necrotic processes induced by the co-incubation of SElX and leukocytes. Accordingly we suggest it is more likely that a targeted mechanism is acting on the neutrophil. Given the broad number of potential glycoprotein receptors that SElX may interact with it is feasible that SElX has multiple effects on the function of the neutrophil and other phagocytes such as macrophages. SElX has already been shown to interact with PSGL-1 albeit at high concentrations (Fevre et al., 2014) and the affinity precipitation analysis in section 4.3.4 suggests additional integrins may be targeted. In this manner it is possible that SElX could contribute to blocking of neutrophil rolling and transcytosis to the site of infection similar to the function exhibited by SSl5 (Bestebroer et al., 2007).

SElY was demonstrated in chapter 4 to bind to neutrophils and monocytes. However SElY did not have any effect on neutrophil phagocytosis of *S. aureus* even at very high concentrations (Fig 5.3) and additional assays are required to investigate the capacity of SElY to interfere with neutrophil function.

S. aureus has been shown to encode at least 25 SAgs (Ono et al., 2015, Spaulding et al., 2013), but to date limited additional functions outside of inducing T-cell proliferation have been reported (Spaulding et al., 2013). Given the array of SAgs encoded by the great

majority of clinical isolates it seems likely that induction of T-cell proliferation is critical to persistence of *S. aureus* in its host, mediated via the activation of a broad range of T-cell Vβ subtypes and subversion of a critical arm of the adaptive immune system (Spaulding et al., 2013). However, the partial redundancy in function of SAgs made by the same strain suggests the possibility of alternative functions. Bi-functional SAgs have been observed in that some of these proteins can induce emesis which may aid the transmission between naïve host (Hu and Nakane, 2014). Other work has shown that SAgs can impact on the innate immune system by manipulating the cytokine expression resulting in T_H17 immune dysregulation (Xu et al., 2014). This mechanism is dependent on inducing T-cell proliferation in different sites in the host, subverting cytokine release and causing immune dysregulation. The retained mitogenicity of the neutrophil binding deficient mutants of SElX demonstrates that SElX can induce T-cell proliferation irrespective of its ability to bind to neutrophils and monocytes. This is an important observation as it demonstrates that the protein mediates its function through, at least, 2 distinct structural sites within the protein. These data identify SElX as the first example of a SAg exhibiting 2 distinct immune evasion functions.

The discovery of a bi-functional SAg correlates with a new appreciation in the field of staphylococcal diseases that many virulence proteins have multiple functions. For example, cellular toxins such as Hla, LukAB and PVL (at sub-lytic concentrations) can subvert cellular functions and contribute to *S. aureus* persistence by inducing inflammation and mediating tissue damage (Davis et al., 2011, Holzinger et al., 2012, Melehani et al., 2015, Kebaier et al., 2012, Munoz-Planillo et al., 2009). The major difference between these examples and SElX is that for the lytic toxins the secondary function is still mediated by the same binding interaction that leads to pore formation and as a result the functional impacts occur in the same cell subset that can be intoxicated. Having distinct binding-tropisms potentially allows SElX to interact with a wider number of cells and impact on a broader

range of immune functions either through direct functional inhibition or interference with immune signalling.

Chapter 6

General Discussion

6.1 Sequence diversity amongst staphylococcal SAg.

SAgs have been shown to play a direct role in severe systemic diseases such as toxic shock syndrome and food poisoning (Spaulding et al., 2013). However SAg genes are harboured by all clinical isolates of *S. aureus* suggesting they play an important role in general pathogenesis of *S. aureus* infection and potentially in colonisation (Omoe et al., 2005, Spaulding et al., 2013, Wilson et al., 2011). Given that these proteins interact with the TCR signalling pathway it is likely that non-specific activation of T-cells can contribute to immunomodulation by *S. aureus*. It has been shown that anergy of memory T-cells can be induced by exposure to SAgs leading to the clonal deletion of memory T-cells (Watson et al., 2012). This may result in a significant deficiency in the ability of the adaptive immune response to respond effectively to infection. A similar process has been observed by the clonal deletion of B-lymphocytes following non-specific stimulation by SpA (Pauli et al., 2014). The wide diversity in the sequence of SAgs is likely to have been generated in response to the diversity of Vβ sub-types of TCR allowing a small complement of SAgs expressed by one isolate to activate a wide array of T-lymphocytes sub-populations. Despite the critical role these genes play in pathogenesis it is possible there is some redundancy of function. This possibility allied to the emerging theme of the multi-functionality of *S. aureus* virulence determinants led us to the hypothesis these proteins may play more than one role in *S. aureus* infections and may exhibit functions distinct from the induction of T-cell proliferation.

In the current study we investigated the structural, physical and functional properties of three staphylococcal SAgs with particular emphasis on the previously characterised SAg SElX. The SAgs SElY and SElZ have recently been identified and to date limited characterisation of their function and role in *S. aureus* pathogenesis has been performed. SElX was selected as it is found in more than 95% of clinical isolates of *S. aureus* and the phylogenetic analysis of the *selx* gene demonstrated that the sequence of this protein is more

similar to the SSl-proteins than the majority of other SAgs (Wilson et al., 2011). SElY was also shown to share a sequence more closely related to the SSl-proteins than the majority of other staphylococcal SAgs. The relation to SSl-proteins is noteworthy as it suggests that SElX and SElY may contain motifs that are also found in the SSl-proteins which may confer immune evasion functions distinct from superantigenicity. Prior to the current project, SElZ had been shown to be mitogenic for bovine T-cell lymphocytes and this has yet to be demonstrated for human T-lymphocytes (Wilson, 2010). Therefore this study set out to determine the functional complement of three staphylococcal SAgs by determining emetic potential, further characterising the mitogenic function of these SAgs and studying any role in the modulation of the innate immune system.

6.2 Staphylococcal virulence determinants can exhibit a broad functional complement.

Examples of multifunctional determinants as described earlier include EAP which can play a role in host cell invasion and inhibit the activity of neutrophil elastase, PVL which has been demonstrated to induce inflammation independent of cell lysis and Cna which, in addition to its role as an MSCRAMM, can bind C1q and block the complement cascade (Kang et al., 2013, Stapels et al., 2014, Holzinger et al., 2012). This is a limited list of examples but all demonstrate a new understanding in the field that many virulence factors exhibit multiple distinct functions.

The apparent functional redundancy exhibited by *S. aureus* with regard to pathogenesis, re-enforced by the multifunctionality of many virulence proteins may allow *S. aureus* to retain infectivity in the face of immune neutralisation of specific virulence determinants. This is an important consideration for the development of vaccines and anti-virulence therapy as creating immunity or blocking the activity to one or two virulence

determinants may prove ineffective if the bacteria can express an alternative protein which performs a similar function to the inactivated factor. Therefore developing an understanding of the full functional complement of critical *S. aureus* virulence determinants will give us the best chance of developing targeted therapies that will by-pass the redundancy intrinsic in these factors and help develop highly effective therapy against *S. aureus* disease.

6.3 Mitogenic activity of SElX, SElY and SElZ.

The superantigenic mechanism of SElX has been previously well described demonstrating potent T-lymphocytes mitogenicity, a Vβ specific T-cell activation profile and enhancement of LPS sensitivity (Wilson et al., 2011). One question that was not addressed completely in this study was how the protein interacted with MHC class II given the truncated OB domain that was predicted. In the current study this question was partly addressed by looking at the ability of SElX to bind zinc cations. It was found that this protein does not interact with zinc and therefore it is unlikely to encode a variant of the high affinity MHC class II binding site. The CD analysis also suggested that SElX contains more α-helical secondary structure than the model suggests. Given the OB domain of the SElX model is predicted to be made up entirely of turns and loops there is a strong indication this model is inaccurate. There are residues in the relative position that could form the low affinity site but given the structural data obtained so far it does suggest a novel MHC class II interaction may be involved in the SAg function of this protein.

SElY has been shown to induce T-cell proliferation in humans previously (Ono et al., 2015) and the mitogenicity analysis performed in this study suggests it has a similar potency to other well characterised SAgs such as SEA and TSST-1. The zinc-binding experiments demonstrated that this SElY does not interact with zinc and therefore is likely to interact with the MHC class II site through the low affinity binding site only. The most

closely related SAg to SElY that has been characterised to date is SET and as suggested by Ono et al. (2015) SElY may share the property of T-cell activation independent of the of Vβ chain of the TCR (Ono et al., 2008a). Screening of the Vβ activation profile of SElY needs to be performed before this characteristic can be confirmed.

SElZ was shown to be mitogenic for human T-cells and the potency of this SAg was found to be at a similar level to that exhibited by TSST-1 and SEA (Fig 3.6). The predicted structure of SElZ suggests this protein encodes two zinc binding sites along with the low affinity binding site for MHC class II. The zinc binding experiments performed in the current study suggests SElZ can bind to zinc although this did not appear to be dose-dependent. This is analogous to SEC which also expresses two zinc binding sites in its structure and contributes to dimerization of the protein and clustering of MHC class II complexes on the surface of an APC (Chi et al., 2002). If SElZ is binding to zinc then it may be used as a cofactor for MHC class II dimerization and it is proposed that the dimerization of MHC class II reduces the clearance of SAg-MHC class II complexes on the surface of the cell and activates the expression of IL-1β and TNF-α from the APC which increases the recruitment of T-lymphocytes to the APC, allowing the SAg to induce T-cell proliferation (Sundström et al., 1996, Mehindate et al., 1995). In the host this mechanism could enhance the stimulation of T-lymphocytes by recruiting them to the activated APC increasing the potency of the SAg during infection.

6.4 Emetic functions of SElX, SElY and SElZ.

Staphylococcal food poisoning is highly prevalent worldwide and given the critical role that enterotoxins play in this disease it is important to identify all emetic proteins secreted by *S. aureus* as this will help to identify strains with food poisoning potential to assist in surveillance and control efforts (Hennekinne et al., 2012, Hu and Nakane, 2014). The current

study has demonstrated that SElX, SElY and SElZ are all highly stable, a property shared with other SAgs including TSST-1 and SEs (Cavallin et al., 2000b, Li et al., 2011). It is proposed that the highly stable structure of SEs allow the proteins to progress through the GI tract and induce emesis (Hu and Nakane, 2014). As SElX, SElY and SElZ all exhibit a high level of stability determination of emetic activity was critical as these three proteins may be novel enterotoxins.

In the house musk shrew model it was found that SElZ and SElX were non-emetic. SElY was found to induce emesis in a study independent of this work (Ono et al., 2015). Given the novel predicted structure for SElX it is unsurprising that this protein does not exhibit emetic activity as it is quite distantly related to other enterotoxins and does not encode a predicted cysteine loop. Of the three proteins tested it was predicted that SElZ would induce emesis given its relatedness to other well characterised SEs and the predicted presence of a cysteine loop. However no emetic activity was seen in the house musk shrew model. This model was chosen as it has many practical benefits that make it more attractive than non-human primate models (Hu et al., 2003). It is possible that SElZ is emetic but that the model is not sensitive enough and it cannot be ruled out that it would be emetic in the primate model. SElY was found to be emetic in the house musk shrew model independently of this study (Ono et al., 2015). Ono et al (2015) reported a lower level of heat resistance compared to SET or SEA which is contrary to our data which suggests SElY exhibits structure with a similar heat resistance to that exhibited by TSST-1. One reason for this discrepancy may be the methods used to assess heat resistance as Ono et al. (2015) used SDS PAGE analysis to determine the degradation of SElY after boiling, reporting complete degradation after 2 h. Despite a lower resistance to heat degradation compared to other SEs, the stability data for SElY obtained in the current study suggests it can retain its structure at higher levels of heat than some SEs such as SEA. Accordingly, SElY could persist in food after improper cooking and pose a food poisoning risk to humans.

6.5 SElX exhibits two distinct immunomodulatory roles.

In the current study for the first time we have identified a SAg, in SElX, that exhibits two distinct immunomodulatory functions. The study demonstrated that this protein shares a glycan-binding motif common in SSl-proteins, and in other bacterial and viral proteins which allow the protein to bind to the surface of neutrophils (Baker et al., 2007). The proteins are still mitogenic even when neutrophil binding is abolished demonstrating the superantigenic function of the protein is distinct from the neutrophil binding function.

SElY was also found to interact with neutrophils and monocytes but unlike SElX this interaction appears to be limited to human cells. Also unlike SElX, SElY binding to neutrophils does not appear to be mediated by the glycan binding domain as this motif is not present in the SElY sequence. Given the SElY-neutrophil/monocyte interaction is unlikely to be glycan-mediated and lower affinity, compared to SElX, it suggests that SElY may be binding to a specific surface protein receptor(s) on the neutrophil and monocyte surface. SElY was tested in a number of functional assays including the phagocytosis assays and no functional impact on neutrophils was observed. SElY displays a slightly higher binding affinity for monocytes than neutrophils so any future work should include the functional impacts of SElY on monocytes and macrophages as well as neutrophils. Despite the lack of a characterised function to date, the leukocyte-binding activity of SElY suggests that the bi-functionality of SAgs may not be limited to SElX and work should be extended to identify which other SAgs exhibit more than one immune evasion function.

The identification of two distinct functions mediated by SElX poses the question of the relative role of these distinct functions in *S. aureus* pathogenesis. The transcriptional analysis of SElX demonstrated that this toxin is regulated by the *saeRS* locus and repressed by the *agr* locus. The *saeRS* regulon is made up of a number of important immune modulators including CHIPS, SCIN and many of the SSl-proteins (Rooijakkers et al., 2006,

Nygaard et al., 2010). Given this co-regulation it suggests that the primary role of SElX could be as an innate immune modulator. To test this hypothesis the mutations that abolish neutrophil binding in rSElX should be introduced into the chromosome of *S. aureus* and tested in an *in vivo* model. If the precise residues involved in superantigenicity can be defined in SElX then site-directed mutagenesis of these residues could be carried out, and the role of SElX superantigenicity examined in the same infection model.

6.6 Potential mechanisms of SElX inhibition of neutrophil inhibition.

The affinity precipitation analysis demonstrated that SElX can potentially interact with a large number of neutrophil proteins and that the binding of SElX may contribute to the inhibition of a number of important cellular processes. The binding partners of SElX determined by the affinity precipitation analysis (Fig 6.1) may be involved in the functional inhibition of neutrophils in the survival and phagocytosis assays. The binding of SElX to the surface of neutrophils allows the protein to play a role in early immune evasion against neutrophils. SElX was demonstrated to inhibit IgG-mediated phagocytosis independent of Fcγ receptor signalling which suggests the mechanism of neutrophil inhibition meditated by SElX is not due to the antagonism of these immune signalling receptors. Therefore an alternative mechanism of inhibition is reducing phagocytosis and this is likely due to activation or repression of other pathways in neutrophils which contribute to the regulation of immune processes in the neutrophil. The binding partners of SElX that were identified during this study include CD45, CD13, CD31, CD148 and CD50. These molecules are all important signalling molecules involved in both neutrophil and T-cell activation. It is possible the blocking of one or all of these receptors can impair mechanisms that require activation during the IgG mediated phagocytosis (Fig 6.1).

The role of CD45 and CD148 have been better characterised in lymphocytes but they have been shown in myeloid cells to play a role in cell adhesion (Hermiston et al., 2009). Bone marrow derived macrophages deficient in CD45 were unable to maintain the adhesion to surfaces (Roach et al., 1997). The activation of myeloid cells is also affected by the deletion of CD45 as mast cells deficient in CD45 do not degranulate properly after IgE cross linking and immune activation by immunoreceptors (e.g. FcγR) is defective in macrophages when CD45 is deleted (Berger et al., 1994, Zhu et al., 2008). The glycoreceptor CD148 activates the same kinase pathways as CD45 and contributes to the regulation of immune cell activation (Hermiston et al., 2009, Zhu et al., 2008). These studies suggests the blocking of with CD45 or CD148 with SElX on the surface of neutrophils could subvert the migration of neutrophils to the site of infection or prevent proper activation of the neutrophil even after stimulation of antigen receptors, such as the Fcγ receptors. Limited data is available on the role of CD45 in neutrophils but work does suggest this receptor is important in the repression of the oxidative burst and is down regulated once a neutrophil is activated (Fialkow et al., 1997). CD45 has been demonstrated to have a role in the regulation of IgG-mediated phagocytosis and can also reduce IgG opsonised particle uptake by dephosphorylating factors that lead to actin polymerisation (Yamauchi et al., 2012). Binding of CD45 to SElX could activate CD45 resulting in the repression of the oxidative burst and IgG mediated phagocytosis.

Another possible mechanism of neutrophil inhibition is that SElX is internalised utilising a similar mechanism exploited by some SSl-proteins like SSl5 and SSl11 (Baker et al., 2007, Chung et al., 2007). Once inside the neutrophil SElX can interfere with internal pathways and promote *S. aureus* survival (Fig 6.1). We have demonstrated that SElX may interact with intracellular proteins as the affinity precipitation analysis revealed a potential interaction with MGAM, a glucamase found in neutrophil granules (Rorvig et al., 2013) and p22-PHOX, a key component in the cytochrome B complex (Dinauer et al., 1990). This idea

is given further weight by the reduced survival of the *S. aureus selx* deletion mutant after co-incubation with human neutrophils. The most likely explanation for the effect of SElX in this assay is intraphagosomal expression of SElX leading to disruption of degradative enzymes like MGAM and interference with factors such as cytochrome B, involved in the oxidative burst (Fig 6.1). The structural analysis of SElX demonstrated that this protein exhibits a high level of structural stability at varied pH therefore it is possible the protein is capable of retaining activity within the low pH environment of the phagosome. However, the expression of SElX within the phagosome in addition to the possible internalisation of extracellular SElX needs to be investigated in future.

In a previous study, SElX was shown to interact at high concentration with the neutrophil integrin PSGL-1, a target shared with SSl5 (Fevre et al., 2014). In the current study we have demonstrated another potential target these proteins share, matrix metallopeptidase 9 (MM9) (Itoh et al., 2010a). Inhibition of this protein could reduce neutrophil migration to the site of infection as MM9 contributes to basement membrane degradation allowing the neutrophil to transcytose through the endothelium of the blood vessel (Itoh et al., 2010a). CD50 (ICAM-3) and CD66c can both interact with CD11/CD18 which form either the MAC-1 or LFA-1 complexes and are integrins which are critical in the activation of migration of leukocytes to the site of infection (Futosi et al., 2013, Skubitz et al., 1996, Campanero et al., 1993). If SElX is interacting with these proteins it is likely the protein is antagonising the activation of these complexes which would impact on the global activation of the cell and prevent immune processes such as phagocytosis and chemotaxis. CD31 is a molecule that can contribute to the rolling of neutrophils along the endothelium, the initial stage of neutrophil transcytosis (Liu et al., 2012). Affinity precipitation analysis suggests that SElX interacts with CD31 as well and may antagonise neutrophil rolling by blocking the binding of CD31 and as previously shown PSGL-1 (Fevre et al., 2014). The inhibition of integrin function and interference with neutrophil migration has been shown to

be a critical function of SSl5 (Armstrong et al., 2012, Bestebroer et al., 2007, Bestebroer et al., 2009) and given SElX has been shown to interact directly with an array of molecules which contribute to neutrophil migration, it is likely SElX contributes to immune evasion through this mechanism in addition to direct inhibition of neutrophil function. Further functional assays need to be performed to determine the effect that SElX has on neutrophil migration given the broad number of targets identified in this study.

The binding candidates of SElX identified in the affinity purification analysis suggest a potential role for SElX in the inhibition of neutrophils efferocytosis by macrophages. When apoptotic processes are activated the neutrophil undergoes changes in the expression of surface glycoproteins. ICAM-3 is cleaved from the surface of an apoptotic neutrophil and acts as a chemoattractant for macrophages and ICAM-3 can also act as an 'eat me' signal to macrophages on the surface of neutrophils (Torr et al., 2012, Kristof et al., 2013). The expression of CD13 is upregulated on the surface of apoptotic neutrophils and is proposed to enhance the degradation of proinflammatory cytokines and reduce inflammation (Hart et al., 2000). ICAM-3 and CD13 have both be shown to be potential binding partners of SElX in the current study. SElX may be able to bind to surface expressed or soluble ICAM-3 preventing this protein from being used as a signal for macrophages. The blocking of CD13 may prevent the degradation of pro-inflammatory signals which result in the continuation of inflammation and the blocking of efferocytosis. *S. aureus* has been shown to be able survive and persist within a neutrophil and to maintain this niche as the bacteria exploits mechanisms to prevent macrophages from removing infected neutrophils (Greenlee-Wacker et al., 2014).

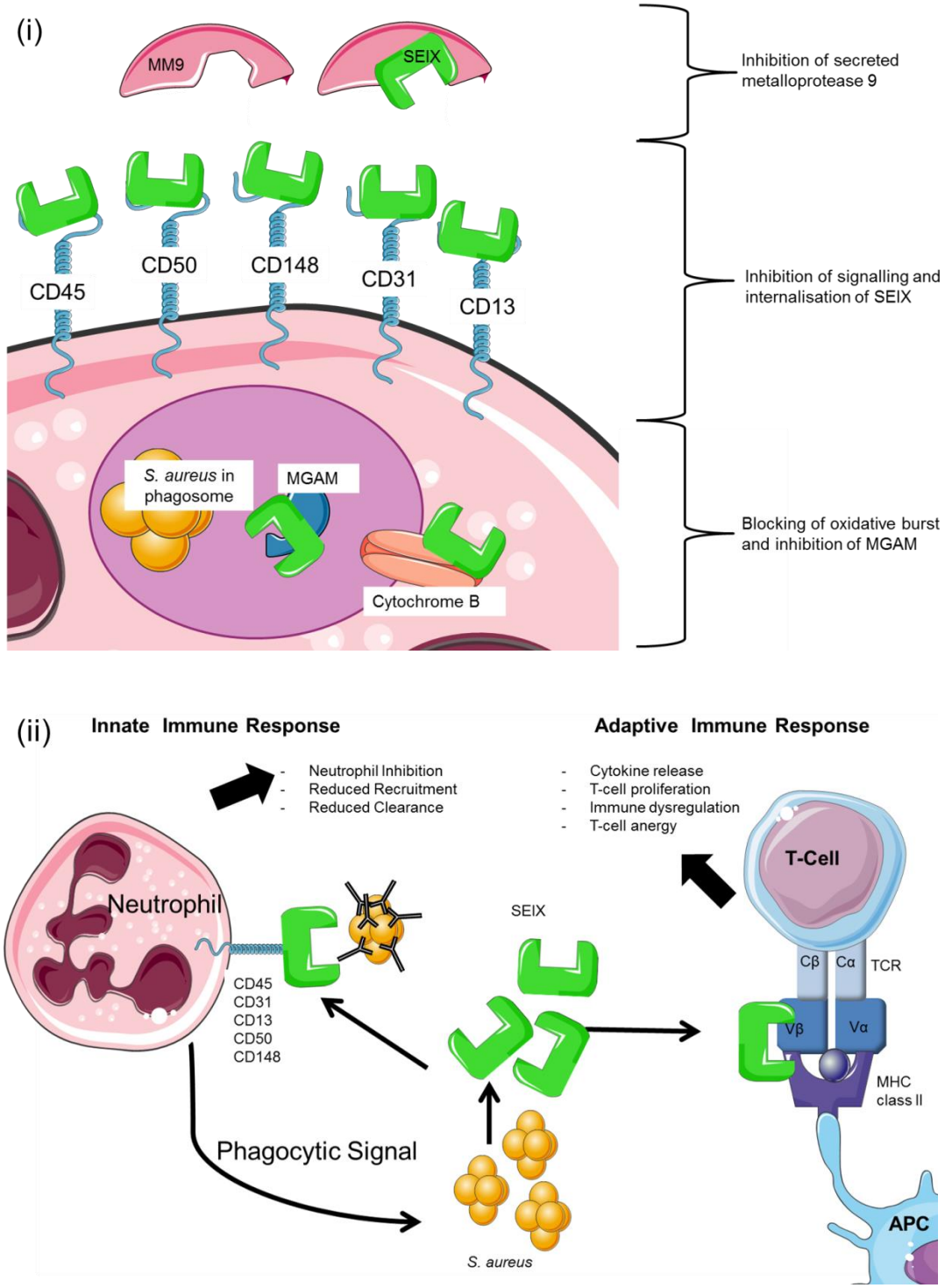


Figure 6.1: Proposed model of SEIX function during *S. aureus* infection. (i) SEIX has been shown to interact with a number of glycoproteins from neutrophils and these interactions could have wide ranging impacts on neutrophil function. (ii) SEIX can interact and manipulate both adaptive and innate arms of the immune system. Figure prepared using images from Servier Medical Art.

6.7 Role of SElX in *S. aureus* pathogenesis.

SElX exhibits distinct functions that can subvert different immune pathways. This insight allows us to develop a model for the role of SElX in *S. aureus* pathogenesis (Fig 6.1). In the current study we have demonstrated that SElX expression is under the regulatory control of the *sae* locus which is activated by signals associated with the phagocytes (Geiger et al., 2008, Voyich et al., 2009). The activation of *sae* results in the expression of SElX which then binds with neutrophils reducing IgG mediated phagocytosis and in turn reduces the clearance of *S. aureus*. The interaction of SElX with neutrophils may affect the neutrophils in other ways as discussed in section 6.6 resulting in reduced recruitment of neutrophils to the sight of infection and enhancing *S. aureus* survival when the bacteria interact with neutrophils. SElX was shown to bind to monocytes and this interaction was shown to be mediated by the sialic acid binding motif which suggests that these monocytes could be inhibited in the same way as neutrophils. Further work needs to be done to understand how SElX interacts with other phagocytes and what impact this can have on *S. aureus* clearance by the immune system.

The stability of SElX may allow the protein to persist at the site of infection and subsequently act as a SAg as the adaptive immune response is recruited, inducing T-cell proliferation and resulting in immune dysregulation (Fig 6.1). As lymphocytes become activated the glycotransferase pathway is upregulated, glycosylating a number of receptors which may provide targets for SElX to bind to and inhibit. Further work should look at repeating the binding assays with activated lymphocytes to determine if SElX can bind to activated T-lymphocytes with high affinity, what the targets of SElX are on the surface of activated lymphocytes and which immune pathways are affected by SElX binding.

6.8 Conclusion

The sequence diversity found among staphylococcal SAgs facilitates interaction with one of the most complex parts of the immune system. However given the chance there is for overlap and redundancy it would be surprising if many SAgs did not exhibit more than one function. In the current study we have demonstrated that SAgs can exhibit functions not related to non-specific T-cell proliferation. SElX has been shown to be the first example of a bi-functional SAg with two distinct immunomodulatory roles in that it can both inhibit neutrophils and subvert T-lymphocyte activation. Given the sequence diversity of SAgs it is likely this bi-functionality is not limited to SElX, and more work needs to be done to further explore the role of SAgs in *S. aureus* pathogenesis.

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Appendix 1

1.1 Amino acid alignment of SElY allelic variants

```
SElY1  MKAKLWFLLT TLAFLIAVTG SIGIA-EVKA KTTGLITENS NDSLKEHYAQ KFEVYTNKEV TGVGENYIDT KVDTYNVRTV LYNTDYLKQF KNQDKVNIWG TLYENQQSKV YRGTVVKYDP ISKVTNLSYR MNLFVNGHQT KVNPDSLLEV  150
SElY2  .......... .......... .....-.... .......... .......... .......... .......... .......... ......F... .......... .......... .......... .......... .......... ..........
SElY3  .......... .........E .....-.... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
SElY4  .......... .........E .....-.... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
SElY5  .......... .......... .....-.... .......... .......... .......... .........V .......... .......... .......... .......... .......... .......... .......... ..........
SElY6  .......... .......... .....-.... .......... .......... .......... .........V .......... .......... .......... .......... .......... .......... .......... ..........
SElY7  .....C.... .......... .....-.... .......... .......... .......... .........A .......... .......... .......... .......... .......... .......... .......... ..........
SElY8  .....C.... .......... .....-.... .......... .......... .......... .........A .......... .......... .......... .......... .......... .......... .......... ..........
SElY9  .....C.... .......... .....-.... .......... .......... .....S.... .........A .......... .......... .......... .......... .......... .......... .......... ..........
SElY10 .....C.... .......... .....-.... .......... .........E .......... .........A .......... .......... .......... .......... .......... .......... .......... ..........
SElY11 .......... .......... .....-.... .......... .......... .......... .........A .......... .......... .......... .......... .........S .......... .......... ..........
SElY12 .......... .......... .....-.... .......... .......... .......... .........A .......... .......... .......... .......... .......... .......... .......... ..........
SElY13 .......... .......... .....-.... .......... .......... .........I .........A .......... .......... .......... .....K.... .......... .......... .......... ..........
SElY14 .......... .......... .....-.... .......... .......... .......... .........A .......... ....G..... .......... .......... ........E. .......... .......... ..........
SElY15 .......... .......... .....-.... .......... .......... .......... .........A .......... .......... .......... .......... .......... .......... .......... ..........
SElY16 .......... ........M. .....-..E. .......... .......... .......... .........A .......... .......... ..K....... .......... .......... .......... .......... ..........
SElY17 .......... .......... ....V-.... ..N....... .......... .......... .........A .......... .......... .......... .......... .......... .......... .......... ..........
SElY18 .......... .......... F....E.... ..N......G .......... N.......... ..I.T....A .......... ........R. ....R..... .......... .......... .......... T......... .....K....
SElY19 .......... .......... F....E.... ..N......G ......Y... N.......... ..I.T....A .......... ........R. ....R..... .......... .......... .......... T......... .....K....
SElY20 .......... .......... F....E.... ..N......G .......... N.......... ..I.T....A .......... ........R. ....R..... .......... .......... .......... T......... .....K....

SElY1  KNKQISLKET DFRIRKYLLE KEHLYSNYNS GELIIEMKNG ARHKIDLGDI LSDSQEKTFD FDNISHIDIY MK 222
SElY2  .......... .......... .......... .......... .......... .......... .......... ..
SElY3  .......... .......... .......... .......... .....Y.... .......... .......... ..
SElY4  .......... .......... .......... .......... .......... .......... .......... ..
SElY5  .......... .......... .......... .......... .....G.... .......... .......... ..
SElY6  .......... .......... .......... .......... .......... .......... .......... ..
SElY7  .......... .......... .......... .......... .......... .......... .......... ..
SElY8  .......... .......... .......... ....V..... .......... .......... .......... ..
SElY9  .......... .......... .......... ....V..... .......... .......... .......... ..
SElY10 .......... .......... .......... ....V..... .......... .......... .......... ..
SElY11 .......... .......... .......... .......... .......... .......... .......... ..
SElY12 .......... .......... .......... .......... .......... .......... .......... ..
SElY13 .......... .......... .......... .......... .......... .......... .......... ..
SElY14 .......... .......... .......... .......... .......... .......... .......... ..
SElY15 .......... .......... .......... .......... E......... .......--- --RLIL.ILV I-
SElY16 .......... .......... .......... .......... T......... .......... .......... ..
SElY17 .......... .......... ..Q..N.... ........S. .......... .......S.. .......... ..
SElY18 ....L..... .......... ..Q..D.... ......L... E..T...... .......S.. .......... ..
SElY19 ....L..... .......... ..Q..D.... .......... E..T...... .......S.. .......... ..
SElY20 ....L..... .......... ..Q..D.... .......... E..T...... .......S.. .......... ..
```

Amino acid alignment of 20 allelic variants of SElY identified in the SHAC dataset. 20 different amino acid sequences were identified and aligned. Dots signify conservation with the corresponding location in SElY1, dashes signify a gap in the sequence alignment. Signal peptide was determined using the SignalP 4.1 web tool (http://www.cbs.dtu.dk/services/SignalP/) and coloured red.

1.2 Amino acid alignment of SElZ allelic variants

```
SElZ1  MRKIFILITL LFGYSSYSLL EARAETQNDP NISELNKSSQ YTGSWHNIWY LYNSDPVNAK KIKLSDKFLS HDFIVPINNP GHYDYVKTEL KDSTMASSFD GKEVDIFGVN YFDQCYFSNE NIQCDSNQGG GSKKTCMYGG ITLNENNTNN  150
SElZ2  .......... .......... ....K..... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
SElZ3  .......... .......... .......... .......... .......... .......... .......... .......... .......... .....T.... .......... .......... .......... .......... ..........
SElZ4  ...V...... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
SElZ5  ...V...... .......... Q.K....... .......... .......... .......... .......... ........S. .......... .......... .......... .......... .........A .......... ..........
SElZ6  ...V...... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........A .......... ..........
SElZ7  ...V...... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........A .......... ..........
SElZ8  ...V...... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........A .......... ..........
SElZ9  ...V...... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........A .......... ..........
SElZ10 ...VL..... .....C.L.. ..K....... .......A.. .......... .......K.. .T........ ........S. .......... .......... .......... ..H....... .........A .......... ..........
SElZ11 ...VL..... .....CNL.. ..K....... .......A.. .......... .......... .......... ........S. .......... .......... .......... ..H....... .........A .......... ..........

SElZ1  RIQPIVVKVY ENDSVTLSFD INIDKETVTI QELDYKVRNK LISKINLYHL GGTSYETGYI KFIENGNRYY WYDMMPDPGF TQSKYLMIYR GNETVESAKT EIEVHLTKK 259
SElZ2  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........
SElZ3  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........
SElZ4  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........
SElZ5  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........
SElZ6  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........
SElZ7  .......... .......... .......I.. .......... .......... .......... .......... .......... .......... .......... .........
SElZ8  .......... .......... .......... .......... .......... .......... .......... .......... ..P....... .......... .........
SElZ9  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .........
SElZ10 .......... .......... .......... .......... .......... .......... .....A.... ........S. .......... .......... .........
SElZ11 .......... .......... .......... .......... .......... .......... .....A.... ........S. .......... .......... .........
```

Amino acid alignment of 20 allelic variants of SElZ identified in the SHAC dataset. 11 different amino acid sequences were identified and aligned. Dots signify conservation with the corresponding location in SElZ1, dashes signify a gap in the sequence alignment. Signal peptide was determined using the SignalP 4.1 web tool (http://www.cbs.dtu.dk/services/SignalP/) and coloured red.

1.3 Amino acid alignment of SElX and TSST-1 showing predicted positions of residues involved in superantigenicity

```
SElX    -STQNSSSVQ DKQLQKVEEV PN-----NSE KALVKKLYDR YSKDTINGKS NKSRNWVYSE RPLNENQVRI HLEGTYTVAG   80
TSST-1  STNDNIKDLL DWYSSGSDAF TNSEVLDNSL GSMRIKNTDG SISLIIFPSP YYSPTFSKGE K-VDLNTKRT KKSQHTSEGT   80

SElX    RVYTPKRNIT LNKEVVTLKE LDHIIRFAHI SYGLYMGEHL PKGNIVINTK DGGK--YTLE SHKELQKDRE N---VKINTA  160
TSST-1  WIHFQISGVT NTEKLPTPIE LPLKVKVHGK DSPLKYWPKF DKKQLAISTL DFEIRHQLTQ IHGLYRSSDK TGGYWKITMN  160

SElX    DIKNVTFKLV KSVNDIEQV* ---------- ------  196
TSST-1  DGSTYQSDLS KKFEYNTEKP PINIDEIKTI EAEIN*  196
```

Amino acid alignment of TSST-1 and SElX showing predicted residues involved MHC class II and TCR binding. TSST-1 and SElX were aligned using the BLOSUM protein weight matrix. MHC class II (**purple**) binding residues in TSST-1 had previously been identified by crystallography studies. The residues predicted to be involved in binding for SElX to MHC class II have been identified based on the residues involved in MHC class II binding in other SAgs including TSST-1. These potential residues were combined with the information regarding the position of MHC class binding amino acids within the TSST-1 protein then using the alignment the most likely MHC class II binding residues within the SElX protein were highlighted. The same approach was adopted to predict the potential TCR binding residues (**yellow**) in SELX.

1.4 Amino acid alignment of SElY and TSST-1 showing predicted positions of residues involved in superantigenicity

```
SElY          KTTGLITENS NDSLKEHYAQ KFEVYTNKEV TGVGENYIDA KVDTYNVRTV LYNTDYLKQF KNQDKVNIWG TLYENQQSKV  [ 80]

TSST-1        -----STNDN IKDLLDWYSS GSDAFTNSEV LDNSLGSMRI KNTDGSISLI IFPSPYYSPT FSKGEKVDLN TKRTKKSQHT  [ 80]


SElY          YRGTVVKYDP ISKVTNLSYR ----MNLFVN GHQTKVNPDS LLEVKNKQIS LKETDFRIRK YLLEKEHLY- -SNYNSGELI  [160]

TSST-1        SEGTWIHFQI SGVTNTEKLP TPIELPLKVK VHGKDSPLKY WPKFDKKQLA ISTLDFEIRH QLTQIHGLYR SSDKTGGYWK  [160]


SElY          IEMKNGARHK IDLGDILSDS QEK-TFDFDN ISHIDIYMK*  [200]

TSST-1        ITMNDGSTYQ SDLSKKFEYN TEKPPINIDE IKTIEAEIN*  [200]
```

Amino acid alignment of TSST-1 and SElY showing predicted residues involved MHC class II and TCR binding. TSST-1 and SElY were aligned using the BLOSUM protein weight matrix. MHC class II (**purple**) binding residues in TSST-1 had previously been identified by crystallography studies. The residues predicted to be involved in binding for SElY to MHC class II have been identified based on the residues involved in MHC class II binding in other SAgs including TSST-1. These potential residues were combined with the information regarding the position of MHC class binding amino acids within the TSST-1 protein then using the alignment the most likely MHC class II binding residues within the SElY protein were highlighted. The same approach was adopted to predict the potential TCR binding residues (**yellow**) in SElY.

1.5 Amino acid alignment of SElZ and SEC2 showing predicted positions of residues involved in superantigenicity and the emetic loop motif

```
SElZ     ETQNDPNISE LNKSSQYTGS WHNIWYLYNS DPVNAKKIKL SDKFLSHEFI VPINNP--SH YDYVKTELKD STMASSFDGK   [ 80]

SEC2     ESQPDPTPDE LHKSSEFTGT MGNMKYLYDD HYVSATKVMS VDKFLAHDLI YNISDKKLKN YDKVKTELLN EDLAKKYKDE   [ 80]


SElZ     EVDIFGVNYF DQCYFLNENI QCDSNQGAGS KKTCMYGGIT LNE--NNTNN RIQPIVVKVY ENDSVTLSFD INIDKETVTI   [160]

SEC2     VDVYGSNYYV NCYFSSK--- --DNVGKVTG GKTCMYGGIT KHEGNHFDNG NLQNVLIRVY ENKRNTISFE VQTDKKSVTA   [160]


SElZ     QELDYKVRNK LISKINLYHL GGTSYETGYI KFIENGNRYY WYDMMPDPG- -FTQSKYLMI YRGNETVESA KTEIEVHLTKK   [241]

SEC2     QELDIKARNF LINKKNLYEF NSSPYETGYI KFIENNGNTF WYDMMPAPGD KFDQSKYLMM YNDNKTVDSK S----------   [241]
```

Amino acid alignment of SEC2 and SElZ showing predicted residues involved MHC class II and TCR binding. SEC2 and SElZ were aligned using the BLOSUM protein weight matrix. In SElZ a number of residues were predicted by the I-TASSER program to be involved in binding zinc (coloured **grey)** and play a role in binding MHC class II through the high affinity site. These residues match the relative location is SEC2. TCR binding residues have been identified in previous crystallography studies for SEC2 these were found to be shared by SElZ (**yellow**). The cysteine (emetic) loop motif was also identified in SElZ and is in a similar location compared to SEC2 (**orange**).