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Structural and functional characterisation of the Staphylococcal superantigen-like protein 11 (SSL11)

Matthew Craig Chung

A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Biological Sciences, The University of Auckland, 2008.
Abstract

*Staphylococcus aureus* is a human pathogen with significant socioeconomic impact. It is the causative agent of many life-threatening disease states, including sepsis, staphylococcal toxic shock syndrome and necrotizing pneumonia. *S. aureus* is associated with 1% of all hospital stays and estimated costs of US$9.5 billion / year (for 2000 and 2001) in the USA alone (Noskin *et al.*, 2005).

Recently, a novel family of virulence factors produced by *S. aureus* was identified, which share sequence and structural homology to the infamous superantigens (Arcus *et al.*, 2002; Williams *et al.*, 2000) – proteins that cause a range of symptoms, including food poisoning and toxic shock. This family of staphylococcal superantigen-like proteins (SSLs) includes 14 members, of which 11 (SSLs 1-11) are closely linked within a mobile genetic element, called a pathogenicity island (Kuroda *et al.*, 2001). To date, the literature suggests that the SSLs exhibit important host immune-evasion functions that are distinct from the superantigens. SSL5 has been reported to inhibit leukocyte rolling, a key process in their recruitment to sites of infection (Bestebroer *et al.*, 2006). SSL7 inhibits serum-mediated killing of bacteria (Langley *et al.*, 2005). This thesis describes the investigation into the structure and function of the related protein, SSL11.

The binding specificity of SSL11 has been identified as the trisaccharide Neu5Acα2-3Galβ1-4GlcNAc, where Neu5Ac is essential for interaction, whereas the NAc sidechain of GlcNAc is not, but is highly favoured. The interaction between the related carbohydrate, sialyl Lewis X (sLe\(^x\), or Neu5Aca2-3Galβ1-4(Fucα1-3)GlcNAc), and SSL11 has been characterised at an atomic level by X-ray crystallography. The dissociation constant of SSL11 binding to sLe\(^x\) was determined by surface plasmon resonance to be in the micromolar to sub-micromolar range. Investigation into the effects of SSL11 on neutrophils reveals that nanomolar concentrations of SSL11 inhibited neutrophil rolling on a P-selectin surface *in vitro*. Additionally, SSL11 was rapidly internalized, exhibited a cytoplasmic distribution and caused changes in actin, visible by 2-D gel analysis.

The characterization of the interaction between SSL11 and the previously-identified ligand, FcαRI (the myeloid immunoglobulin A receptor), is also described. The dissociation constant
of SSL11 binding to FcαRI was determined by surface plasmon resonance to be in the micromolar to sub-micromolar range and was found to be sialic acid-dependent. Binding of SSL11 to FcαRI inhibited the binding of its native ligand, IgA.

The sialic acid-dependent glycan-binding site of SSL11 is the sole site of interaction with the neutrophil surface, shown by neuraminidase treatment of cells and complete abrogation of binding by a site-directed functional knockout mutant. Residues involved in glycan binding were found to be conserved in SSLs 2-6, giving rise to a potential related function between this subset of the SSL family.
Acknowledgements

I owe gratitude to so many people that have contributed to this thesis in many different ways. I wish to start by thanking Professors Ted Baker and John Fraser for giving me the opportunity to work on this project. Thank you for your support, direction and enthusiasm. I have enjoyed this experience and am grateful for your guidance.

To the Fraser lab, past and present - It has been a blast. A particular mention goes to the “old school” PhDs – Jace, Lily, Natasha, Nikki and Amanda – thanks for the good times. It would have been far less enjoyable without your company. To the post-docs – Thomas, Birgit, Ries, Indira, Grant and Fiona – you guys have such broad experience and I have benefited greatly from that, so thank you. Thanks to Phil for teaching me the ways of the BIAcore and to Vanessa and Fiona for keeping the place up and running (and making sure I wear my labcoat).

Thanks to the Baker group for putting up with me. In particular, special thanks to Heather for helping me so much with the crystallography side of things. Tom, Fasseli, Chris, David, Jerome and Ghader, thank you for your technical assistance.

To those people in far off places: Bruce Wines, thanks for your help, ideas and continual optimism. Thank you Chris Kirton, you were instrumental in helping me develop the rolling assays.

To my loving family, especially my parents, I couldn’t have done it without your continual and unwavering support. What more can I say but thank you for always being there for me. Mum and Dad, you must be glad that I’m finally on my way to joining the “real world”!

Go team toxin!

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Abbreviations

SI prefixes
n nano- ($10^{-9}$)
μ micro- ($10^{-6}$)
m milli- ($10^{-3}$)
k kilo- ($10^{3}$)

SI units
Å Ångström ($10^{-10}$ m)
s second
min minute
h hour
L litre
g gram

Other units / abbreviations
°C degrees Celsius
2-D two-dimensional
Abs absorbance
ADP adenosine diphosphate
APES 3-aminopropyl triethoxyilane
ATP adenosine triphosphate
bp base pair
BSA bovine serum albumin
CD cluster of differentiation
CFG Consortium for Functional Glycomics
CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHIPS chemotaxis inhibitory protein of *S. aureus*
CHO Chinese hamster ovary
cv column volume
Da Dalton
DAPI 4',6-Diamidino-2-phenylindole dihydrochloride
DNA deoxyribonucleic acid
dNTP deoxynucleotide triphosphates
<table>
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<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>ET</td>
<td>exfoliative toxin</td>
</tr>
<tr>
<td>Fc</td>
<td>immunoglobulin crystallizable fragment</td>
</tr>
<tr>
<td>FcαRI</td>
<td>myeloid immunoglobulin A Fc receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>fast performance liquid chromatography</td>
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<td>glycoprotein</td>
</tr>
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<tr>
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<td>monoclonal antibody</td>
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<td>membrane attack complex</td>
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<td>neutrophil extracellular trap</td>
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<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>OB</td>
<td>oligosaccharide- / oligonucleotide- binding</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pdb</td>
<td>protein data bank</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PECAM</td>
<td>platelet / endothelial cell adhesion molecule</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethyleneglycol</td>
</tr>
<tr>
<td>pl</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonfyl fluoride</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>PVL</td>
<td>panton-valentine leukocidin</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>Req</td>
<td>response at equilibrium</td>
</tr>
<tr>
<td>RFU</td>
<td>relative fluorescence units</td>
</tr>
<tr>
<td>rmsd</td>
<td>root mean square difference</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium-1640</td>
</tr>
<tr>
<td>rsFcαRI</td>
<td>recombinant soluble myeloid immunoglobulin A Fc receptor</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RU</td>
<td>response units</td>
</tr>
<tr>
<td>SAg</td>
<td>superantigen</td>
</tr>
<tr>
<td>Sak</td>
<td>staphylokinase</td>
</tr>
<tr>
<td>SCIN</td>
<td>staphylococcal complement inhibitor</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>staphylococcal enterotoxin</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SET</td>
<td>staphylococcal exotoxin-like toxin</td>
</tr>
<tr>
<td>Sia</td>
<td>sialic acid – N-acetylmuraminic acid / Neu5Ac</td>
</tr>
<tr>
<td>sIgA</td>
<td>secretory immunoglobulin A</td>
</tr>
<tr>
<td>sLe(^\alpha)</td>
<td>sialyl Lewis X / Neu5Ac(\alpha)2-3Gal(\beta)1-4(Fuc(\alpha)1-3)GlcNAc</td>
</tr>
<tr>
<td>SpA</td>
<td>staphylococcal protein A</td>
</tr>
<tr>
<td>Spe</td>
<td>Streptococcus pyrogenic enterotoxin</td>
</tr>
<tr>
<td>SSL</td>
<td>staphylococcal superantigen-like</td>
</tr>
<tr>
<td>SSM</td>
<td>secondary structure matching</td>
</tr>
<tr>
<td>SSSS</td>
<td>staphylococcal scalded skin syndrome</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TeR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TCRS</td>
<td>two component regulatory system</td>
</tr>
<tr>
<td>TNF(\alpha)</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)propane-1-3-diol</td>
</tr>
<tr>
<td>TrxA</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TSST-1</td>
<td>toxic shock syndrome toxin-1</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
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</table>
Chapter 1 - Introduction

1.1 Overview

*Staphylococcus aureus* is a gram-positive bacterium and is the causal agent of many human diseases, including boils, impetigo, abscess and the more serious staphylococcal scalded skin syndrome, necrotizing fasciitis and toxic shock syndrome. An estimated 20% of the human population are persistent carriers, up to 60% are intermittent carriers, and the remaining 20% are persistent non-carriers (Peacock *et al.*, 2001).

In less than 3 years after the introduction of penicillin as a treatment, resistant strains of *S. aureus* began to emerge. The introduction of meticillin (previously known as methicillin) in 1959 to treat penicillin-resistant strains was also a short-lived victory as meticillin-resistant *S. aureus* (MRSA) strains were reported only two years later. Treatment of *S. aureus* has become increasingly difficult, with strains becoming resistant to multiple antibiotics including penicillins, macrolides and aminoglycosides. Due to the high prevalence and often serious nature of this pathogen, it is imperative to develop more effective drugs / vaccines.

Recently, a novel family of genes were identified within a pathogenicity island, thought to be a mobile element and transmissible in phage-like particles. They encode the staphylococcal superantigen-like (SSL) proteins. The SSLs are thus named, as they share consensus sequences found in staphylococcal superantigens (SAgs), such as toxic shock syndrome toxin-1 (TSST-1). The structural fold of the SSLs is similar to those of bacterial SAgs, both comprising an N-terminal oligosaccharide / oligonucleotide-binding (OB) domain (a 5-stranded mixed β-barrel) and a C-terminal β-grasp domain (antiparallel β-sheet packed against an amphipathic α-helix). However, their functions appear to be quite different. This thesis is a synopsis of results from the investigation of SSL11, a novel carbohydrate-binding protein and potential therapeutic target.
1.2 *Staphylococcus aureus*

1.2.1 Staphylococci
Staphylococci are spherical-shaped, gram positive bacteria that form grape-like clusters. They infect a wide host range and survive for long periods outside the host environment. Rosenbach (1884) described two staphylococci by their colour – white (S. *albus* – which has since been renamed S. *epididimis*) and orange (S. *aureus*). Typically, S. *aureus* is hemolytic on blood agar plates, while S. *epididimis* is non-hemolytic. S. *epididimis* is coagulase-negative, while S. *aureus* produces the adhesin coagulase, which causes blood to clot and allows the bacteria to coat themselves with fibrin, making them more resistant to phagocytosis (Hale and Smith, 1945).

1.2.2 Pathogenesis of *S. aureus*
S. *aureus* is a common human pathogen that colonises nasal passages, the throat, and skin. It is the causal agent of many disease symptoms. At the milder end, S. *aureus* can cause food poisoning and superficial skin lesions including boils, abscess and impetigo. More serious effects include the life-threatening septic arthritis, endocarditis, Staphylococcal scalded skin syndrome and toxic shock syndrome (Lowy, 1998).

1.2.3 The *S. aureus* genome and antibiotic resistance
Recently, several *S. aureus* genome sequencing projects have been completed, which include a typical lab strain (Gillaspy *et al.*, 2006), meticillin-resistant *S. aureus* (MRSA) isolates (Baba *et al.*, 2002; Diep *et al.*, 2006; Gill *et al.*, 2005; Holden *et al.*, 2004; Kuroda *et al.*, 2001), a vancomycin-resistant *S. aureus* (VRSA) isolate (Kuroda *et al.*, 2001), a meticillin-sensitive *S. aureus* (MSSA) isolate (Holden *et al.*, 2004), and a bovine strain (Herron-Olson *et al.*, 2006). There are also two partial genomes available for a vancomycin-sensitive strain (Copeland *et al.*, 2006a) and a vancomycin-resistant strain (Copeland *et al.*, 2006b).

Many of the bacterial resistance markers are carried within a mobile genetic element (Staphylococcal chromosome cassette - SCCmec) which permits horizontal transfer between strains. The ability of *S. aureus* to transfer and integrate these resistance markers has given rise to the alarming increase in antibiotic resistance since their introduction as therapeutics. Mobile genes encoding proteins that confer antibiotic resistance include *mecA* (encoding
penicillin-binding protein 2’), bleO (bleomycin resistance protein), ant(9) (spectinomycin resistance protein) and a gene encoding a Far1 homologue (Fusidic acid resistance) (Holden et al., 2004; Kuroda et al., 2001). Other resistance factors are contained on plasmids, e.g. blaZ (β-lactamase), cadDX (cadmium resistance) and arsRBC (arsenate resistance) (Holden et al., 2004; Kuroda et al., 2001).

1.3 Host defenses

The majority of pathogens are unable to penetrate intact skin and mucosal surfaces such as the nasal cavity and gut. Those that manage to invade tissue or blood are generally targeted by multiple protective mechanisms as discussed below.

1.3.1 The complement system

The complement system is a central component of the innate immune system and has been widely reviewed (Gasque, 2004; Kinoshita, 1991). It comprises three inter-connecting pathways – the classical, lectin, and alternative pathways (figure 1.1). Recently, it has been suggested that there may be an additional C3-independent complement activation pathway that appears to utilise thrombin as a C3 substitute (Huber-Lang et al., 2006). Although the complement system is best known for the formation of the membrane attack complex (MAC), it has been firmly implicated in many additional immune responses such as inflammation, phagocytosis, adaptive immunity and developmental processes, (Barrington et al., 2001; Mastellos et al., 2005; Morgan et al., 2005).
i) The three activation pathways

The complement C1 complex is made of six C1q, two C1r and two C1s molecules. The C1 complex binds the Fc portion of antibodies (typically IgG or IgM) bound to pathogens and markers on bacterial surfaces, including lipopolysaccharides (LPS). In the classical pathway, the binding of the C1q subunit to Ig triggers a conformational change that releases C1r, which subsequently cleaves and activates the proenzyme C1s. C1s mediates the cleavage of C4 into the anaphylotoxin C4a and the membrane-associated C4b (Sim and Reid, 1991). When C1s interacts with C4b on the membrane, it initiates the cleavage of C2 into C2a and C2b. C2a complexes with C4b on the membrane, forming the C3 convertase C4b2a, an enzyme that cleaves C3 into C3a and C3b. C3a is a potent anaphylotoxin and C3b forms a trimeric complex with C4b2a (forming C4b2a3b) which is a C5 convertase, an enzyme that cleaves C5 into C5a and C5b.

The lectin pathway recognises arrays of terminal mannose residues on bacterial surfaces (Jack et al., 2001). Upon binding to these carbohydrates, mannose binding protein (MBL)
interacts with MBL-associated serine proteases 1 and 2 (MASP1, MASP2), which are able to directly activate C2, C3 and C4 (Matsushita et al., 2000; Vorup-Jensen et al., 1998) similar to the classical pathway.

In the alternative pathway, C3 is hydrolysed directly on the surface of the pathogen, resulting in deposition of C3b. Factor B then binds to the C3b and this complex is cleaved by Factor D, forming the C3 convertase, C3bBb. C3bBb is stabilised by the binding of properdin and cleaves serum C3 into C3a and C3b, forming the C5 convertase complex, C3bBbC3b.

ii) Formation of the membrane attack complex (MAC)
C5 is cleaved by C5 convertase, generated through one of the three complement pathways, into the potent anaphylotoxin C5a and the membrane-bound C5b. C5b is a receptor for C6 and C7, which stabilise it in the trimeric complex, C5b67. Binding of C8 to C5b67 initiates membrane leakage and is able to lyse cells without C9. C8 induces the polymerisation of C9, completing the MAC, C5b6789 (Podack, 1984; Podack and Tschopp, 1984), a hollow cylindrical pore that allows the leakage of electrolytes and water across the membrane, ultimately leading to osmotic lysis.

iii) The anaphylotoxins, C3a, C4a and C5a
During the complement cascade, small effector peptides are released from C3, C4 and C5 named C3a, C4a and C5a respectively. The C-terminal portions of the peptides are highly conserved and solely responsible for the anaphylotoxic effects, although longer peptides are more efficient at stimulation (Ember and Hugli, 1997). In C3a, the C-terminus is fully conserved across species and function is maintained even in invertebrates (Pasupuleti et al., 2007). These anaphylotoxins elicit many effects including histamine release from mast cells, smooth muscle contraction and increased vascular permeability (Cochrane and Muller-Eberhard, 1968; Gorski et al., 1979). Although all three peptides are closely related, there are some functional differences between them. Both C3a and C5a also act as chemoattractants (Hartmann et al., 1997), but C4a does not (Gorski et al., 1979). C3a and C4a also exhibit direct antimicrobial properties that are effective against both gram-negative and gram-positive bacteria, but C5a does not (Nordahl et al., 2004; Pasupuleti et al., 2007).
1.3.2 Pro-inflammatory responses

Upon stimulation by bacterial products (i.e. LPS and exotoxins), the body reacts with local inflammatory responses. These include monocyte production of the cytokine, tumor necrosis factor alpha (TNFα), and the chemokine, interleukin 8 (IL-8). For example, extracellular protein A, α-toxin, TSST-1, and staphylococcal enterotoxins A and B (SEA, SEB) produced by *S. aureus* are able to induce TNFα production and secretion by keratinocytes (Ezepchuk *et al.*, 1996). Signaling by TNFα can result in the upregulation of IL-8 expression in numerous cell types, including endothelial cells, by up to 100-fold and bacterial products can directly increase secretion by 5-10 fold (reviewed by Hoffmann *et al.*, 2002). These pro-inflammatory signals recruit leukocytes from the blood stream to the site of inflammation to neutralise the pathogen.

1.3.3 Leukocyte recruitment to sites of inflammation

In response to local inflammatory signals, endothelial cells rapidly mobilise P-selectin (CD62P) from internal stores (Weibel-Palade bodies) to the plasma membrane (Hattori *et al.*, 1989), initialising the leukocyte adhesion cascade. The cascade is a multi-step process where leukocytes are stimulated by chemical signals to slow by rolling along endothelium, adhere, and finally transmigrate through the blood vessel into the inflamed tissue (figure 1.2). Because this process is sequential, the inhibition or malfunction of a single step results in impaired immune processes. Examples of genetic disorders that result in impaired recruitment are the extremely rare leukocyte adhesion deficiencies (LAD). LAD type I is caused by defects in CD18 (see section 1.3.3 ii), while LAD type II is caused by impaired fucosylation, a process required for functional modification of the selectin ligand, sialyl Lewis X (sLe^x^ or CD15s – see section 1.3.3 i) (Bunting *et al.*, 2002). Recently, a third form of LAD has been described (type III), which results from a defect in the activation of RapI, a key integrin-signalling molecule (Etzioni and Alon, 2004).

Other factors are involved in leukocyte recruitment, including chemotaxis of neutrophils directly by complement C5a and indirectly via eosinophil stimulation by complement C3a (Daffern *et al.*, 1995; Ehrengruber *et al.*, 1994). Chemotaxis can also occur directly as a result of bacterial products, including formylated peptides (Cicchetti *et al.*, 2002).
i) Leukocyte tethering and rolling

P-selectin glycoprotein ligand-1 (PSGL-1, or CD162) is a 120 kDa receptor expressed as a homodimer on leukocyte surfaces (Moore et al., 1992). It expresses the O-linked, terminal tetrasaccharide sLe\(^\text{a}\), which binds to selectins on vascular endothelial cells with high affinity. There are three types of selectin; P-, L- and E- selectin (CD62P, CD62L and CD62E respectively). P-selectin is the primary selectin involved in leukocyte tethering immediately after tissue trauma, with L-selectin becoming predominant after more than 60 min (Ley et al., 1995). Although E-selectin binds to PSGL-1, their interaction is not required for neutrophil rolling (Yang et al., 1999). In fact, while P- and L-selectin mediate a fast-to-slow rolling of leukocytes, E-selectin appears to be involved in the transition from slow rolling to firm arrest (Smith et al., 2004). The result of PSGL-1 interacting with the selectins is the initial tethering and slowing of leukocytes by rolling on endothelial surfaces, allowing for additional receptor interactions.
ii) Leukocyte adhesion
As the leukocytes roll over the endothelium, they are exposed to IL-8 expressed by endothelial cells at sites of local inflammation. Neutrophils are activated by IL-8 (Sengelov et al., 1993) and the crosslinking of selectins (Simon et al., 1995), which stimulates them to adhere to the endothelium. Adhesion of neutrophils to endothelial cells occurs in two stages. The first stage is the capture of neutrophils from rolling to stasis through the interaction of the β2-integrin lymphocyte function-associated antigen-1 (LFA-1, or CD11a/CD18), with the endothelial cell-bound intercellular adhesion molecule 1 (ICAM-1, or CD54) (Dustin and Springer, 1988). The second stage is the stabilisation of the cell adhesion through the additional interaction of the related macrophage-1 antigen (Mac-1, or CD11b/CD18) with ICAM-1 (Diamond et al., 1991; Hentzen et al., 2000).

Unstimulated neutrophils are able to bind to ICAM-1 through an LFA-1-dependent association (Smith et al., 1989). Activation of neutrophils by IL-8 or binding to ICAM-1 induces a rapid conformational change in LFA-1 in the I-domain allosteric site (IDAS), resulting in a switch into a high affinity state and redistribution in the membrane into clusters (Cabanas and Hogg, 1993; Lum et al., 2002). However, this effect is transient and LFA-1 reverts to the low affinity state within ~30 s, while Mac-1 continues to contribute to the stable binding of neutrophils to endothelial cells for several minutes (Neelamegham et al., 1998).

Neutrophil activation by IL-8 results in the mobilisation of internal stores of the β2-integrin, Mac-1, to the cell membrane (Petrequin et al., 1987; Sengelov et al., 1993). Although there is a 10-fold increase of Mac-1 receptors on the cell surface (Sengelov et al., 1993), only a small population of these are “active” and able to bind to the endothelial cell-expressed ICAM-1 (Diamond and Springer, 1993). This population may be activated by IL-8 (Detmers et al., 1990) or crosstalking with P-selectin (Evangelista et al., 1996) and is thought to occur as a result of a conformational change in the C-terminal domain of CD11b (Xia et al., 2002).

Adhered leukocytes interact with docking structures on the endothelial cells which consist of “cup-like” protrusions that are enriched for ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), LFA-1 and cytoskeletal molecules such as vimentin (Barreiro et al., 2002; Nieminen et al., 2006; Shaw et al., 2004). Luu and colleagues (1999) showed that neutrophils...
move slowly across an endothelial cell monolayer (~8 μm/min) for 20-200 s, before passing through – a process that takes around 60 s.

iii) Extravasation / diapedesis
The process by which leukocytes migrate through the endothelial cell layer into the site of inflammation is known as extravasation or diapedesis. Unlike leukocyte tethering and rolling, this particular component of leukocyte recruitment is less well understood. Leukocytes can migrate by squeezing between adjacent cells (transcytotic) or through individual cells (paracytotic), although the molecular basis that decides which pathway the leukocytes use is unknown (figure 1.3). Many of the processes involved in diapedesis have been found using inhibitory antibodies and arrest of leukocytes at various stages of diapedesis.

Figure 1.3 - Diapedesis
Summary of events that occur in the two types of diapedesis (modified from Petri and Bixel, 2006).

iv) Paracellular migration
After leukocytes migrate on the endothelial cell surface, the first receptor involved is the platelet/endothelial cell adhesion molecule 1 (PECAM-1). This was found by pre-treatment of monocytes or endothelial junctions with a monoclonal antibody (mAb) against PECAM-1.
and the observation that monocyte diapedesis was inhibited by 70-90% (Muller et al., 1993). The monocytes accumulated at the apical surface at the intercellular junctions of the endothelial cells, indicating no effect on leukocyte adherence, but a profound effect on diapedesis. Another molecule, CD99, was recently found on endothelial cells and a 90% inhibition of monocyte diapedesis was observed by blocking with anti-CD99 mAb (Schenkel et al., 2002). The monocytes were arrested when partially through the junction, indicating that CD99 acts sequentially to PECAM-1.

Signalling by cytokines and interferon-γ (INFγ) also causes substantial changes in the endothelium to promote paracellular migration. TNFα is released by activated macrophages, which stimulates natural killer (NK) cells to release INFγ (Roitt et al., 1998). Treatment of endothelial cells with TNFα and INFγ results in a redistribution of junctional adhesion molecules (JAM) (Ozaki et al., 1999) from intracellular junctions. The ligands for JAM-A and JAM-C have been identified as LFA-1 and Mac-1 respectively (Ostermann et al., 2002; Santoso et al., 2002). Additionally, it has been shown that JAM-B on endothelial cells can interact with JAM-C on lymphocytes (Arrate et al., 2001). It is hypothesized that the JAM molecules redistribute from the junctions and interact with receptors on the immobilised leukocyte.

Vascular-endothelial cadherin (VE-cadherin) is a 140 kDa glycoprotein that localises at the intercellular junctions of endothelial cells. Redistribution of VE-cadherin is observed from the intercellular junctions upon treatment with TNFα and INFγ (Lampugnani et al., 1992). The result is an increased permeability of macromolecules across endothelial cell monolayer, an effect shared by treatment of endothelial cells with a mAb against VE-cadherin (Lampugnani et al., 1992).

v) Transcellular migration
Little is currently known about transcellular migration. However, caveolae appear to play an important role. Caveolae have been implicated in numerous processes including endocytosis and numerous cell signal transduction events (reviewed by Parton and Simons, 2007). Caveolin, an integral membrane protein of caveolae, localises at the site of leukocyte docking (Carman and Springer, 2004). Perhaps the most comprehensive study on transcellular migration was published by Millan and colleagues (2006), who show that ICAM-1 is actively
translocated to filamentous actin- and caveolin-rich regions and transcytosed in caveolae. Additionally, they showed that knockdown of caveolin in human umbilical vein endothelial cells (HUVEC) by small interfering (si)RNA specifically reduced transcellular- but not paracellular-migration of T-lymphoblasts.

1.3.4 Neutrophil killing of bacteria

Once neutrophils have migrated to the site of infection, they initiate a plethora of processes aimed at destroying the invading pathogens. These include both intracellular and extracellular pathways as described below.

1.3.5 Phagocytosis

Phagocytosis is the process by which microbes or particles are engulfed by professional phagocytic cells, namely neutrophils and macrophages. This process is enhanced if the particle is opsonised (i.e. coated) with either Igs or complement C3bi. Complement C3bi is produced by the removal of a 2 kDa peptide (C3f) from the α-chain of C3b by Factors H and I (Muller-Eberhard, 1988). Therefore, the primary receptors involved in opsonophagocytosis are the Ig receptors including the FcγR family (Bharadwaj et al., 2001) and FcαRI (Gorter et al., 1987), and the complement C3bi receptor, Mac-1 (Agramonte-Hevia et al., 2002). Signaling through ligation of Fcγ receptors initiates a complex pathway that ultimately results in the redistribution of F-actin, forming pseudopodia that engulf the particle (Greenberg and Grinstein, 2002). The particle is then internalised in a phagosome, or “food vacuole”.

The phagosome undergoes fusion with various secretory vesicles and granules in a process called phagosome maturation, which leads to the killing of internalised bacteria. Four types of granules have been identified in neutrophils, which have been reviewed by Segal (2005) and Lee et al. (2003). The primary (azurophil) granules contain many antimicrobial proteins and peptides, including proteases, lysozyme, myeloperoxidase (MPO), defensins and proteins that permeabilise bacteria. The secondary (specific) granules contain apo-lactoferrin (see section 1.3.7), lipocalin and the majority of lysozyme. Tertiary (gelatinase) granules contain gelatinase but not lactoferrin. Lysosomes contain acid hydrolases and fusion with the phagosome results in the formation of a phagolysosome, an acidic compartment (pH ≤5.0) optimised for the activity of the antimicrobial enzymes.
1.3.6 Oxidative burst

Also known as respiratory burst, oxidative burst is an important anti-microbial process in phagolysosomes. Flavocytochrome b$_{558}$ is a heterodimer of p22$^{phox}$ and gp91$^{phox}$ and is a key component in this process. It acts as an electron pump, using NADPH in the cytosol to generate reactive O$_2^-$ inside the vacuole (Rotrosen et al., 1992). The O$_2^-$ anion can react to form peroxide (H$_2$O$_2$) and the highly reactive hydroxyl radical (OH$^-$). H$_2$O$_2$ is also a substrate for MPO, which catalyses the oxidation of halides that react with and kill bacteria (Hampton et al., 1996).

1.3.7 Lactoferrin

Neutrophils produce and secrete lactoferrin, a non-heme, iron-binding protein with dual function. The first is to chelate all free iron in the site of infection, as iron is an important growth factor for many bacteria (Bezwoda and Mansoor, 1989; Bullen et al., 1978). The crystal structure has been solved (Norris et al., 1991) and reveals an iron binding site in each of the two domains. Lactoferrin is referred to as apo-lactoferrin in its iron-free state and holo-lactoferrin in its iron-bound state. In response to iron chelation by lactoferrin, some bacteria have evolved mechanisms to overcome this, by stealing back the iron by producing siderophores (Crosa, 1989), or expressing lactoferrin receptors on their surface (Chierici and Vigi, 1994; Husson et al., 1993). One mechanism to subvert iron chelation by bacteria, is the sequestration of siderophores by lipocalin-2 (Flo et al., 2004), a protein produced by neutrophils.

Second, lactoferrin is capable of direct binding to porins on the membrane of gram-negative bacteria. This induces the rapid release of LPS, which is associated with an increased susceptibility to anti-bacterial mechanisms (Gado et al., 1991; Leitch and Willcox, 1998). Direct binding occurs via the N-terminus, a highly cationic sequence, distinct from the C-terminal region which is involved in iron chelation (Baker and Baker, 2005). Cleavage of lactoferrin by pepsin results in the release of the N-terminal peptide, lactoferricin, a 25 amino acid peptide containing two internal disulphide bonds (Bellamy et al., 1992). Lactoferricin has been shown to have anti-microbial, -viral, -parasitic and -tumour effects (reviewed by Gifford et al., 2005).
1.3.8 Neutrophil extracellular traps (NETs)

Neutrophils are able to ensnare and kill bacteria extracellularly by releasing NETs. These consist of fibres containing neutrophil granule proteins, chromatin, DNA and proteases such as elastase and their production can be stimulated by IL-8 (Brinkmann et al., 2004). Elastase can degrade bacterial virulence factors (Weinrauch et al., 2002).

1.4 Host evasion by S. aureus

Due to the plastic nature of its genome, S. aureus has evolved and acquired many genes that encode proteins used to evade and subvert the human immune system. Some of these factors will be discussed below, with their relation to, and effect on host immunity.

![Figure 1.4 - Inhibition of the Complement system](image)

Summary of proteins produced by S. aureus that inhibit complement activity.
1.4.1 Complement evasion by *S. aureus*

Because *S. aureus* is a gram positive bacteria, its thick cell wall generally protects it from direct lysis by MAC. However, intermediary steps of the complement cascade are still important for bacterial recognition by neutrophils. Therefore, *S. aureus* has evolved / acquired several anti-complement factors that target key steps, as discussed below. Figure 1.4 summarises the steps within the complement cascade that are inhibited by *S. aureus* proteins.

i) Inhibition of the initiation of the complement system

Staphylokinase (Sak) is a 16 kDa protein whose expression is controlled by the *agr* promotor (Arvidson and Tegmark, 2001). Sak lacks enzymatic activity on its own, but forms an active complex when it binds 1:1 with plasminogen from human serum (Parry *et al.*, 2000). The complex effectively acts like activated plasmin, an enzyme that is able to dissolve clots and cleave extracellular matrix proteins (Vassalli *et al.*, 1991) as well as the hinge region of IgG (Harpel *et al.*, 1989). Rooijakkers and colleagues (2005b) have shown that addition of Sak and plasminogen cleaved IgG bound to the surface of *S. aureus*, in vitro. Because C1q binds to the Fc portion of IgG, cleavage of surface-bound IgG would abolish the activation of C1 in the classical pathway (Duncan and Winter, 1988). *S. aureus* can bind directly to plasminogen, which can be converted to plasmin (Kuusela and Saksela, 1990), indicating a potential role in invasive pathogenesis (Lottenberg *et al.*, 1994).

Staphylococcal Protein A (SpA) was identified as an IgG-binding protein by Forsgren and Sjoquist (1966). The gene encoding SpA, *spa*, is controlled by the *agr* promoter (Huntzinger *et al.*, 2005) and was first identified and partially sequenced by Lofdahl and colleagues (1983). The full sequence reveals an LPXTG motif (Finck-Barbancon *et al.*, 1992), which is the target sequence for sortases to covalently attach the protein to the cell wall (Kruger *et al.*, 2004). SpA binds to the Fc portion of IgG, which performs dual functions; first to inhibit the binding of C1 to the bacterial surface and second to inhibit opsonophagocytosis. SpA may also be released extracellularly (Lindmark *et al.*, 1977) and can stimulate apoptosis of B cells by binding to the V_{H3} region of IgM (Goodyear and Silverman, 2003).

In addition to SpA, another protein produced by *S. aureus* with similar binding specificity was identified and named Staphylococcal IgG-binding protein, or Sbi (Zhang *et al.*, 1998). Although this protein lacks the LPXTG motif (Kuroda *et al.*, 2001), it appears to be membrane-associated. The binding of IgG to surface-bound Sbi is an example of functional
redundancy, as SpA-negative mutants show only slightly less virulence than wild-type strains (Patel et al., 1987). In addition to IgG binding, Sbi also binds to β2-glycoprotein I (Zhang et al., 1999), which shares structural homology with complement factor H, the Ba fragment of complement factor B, and C4-binding protein (Kristensen et al., 1986). Therefore, Sbi could potentially have multiple effects on the complement system.

ii) Inhibition of C3 convertase
Staphylococcal complement inhibitor (SCIN) is a 10 kDa secreted protein that was first identified by Rooijakkers and colleagues (2005a). The gene is found in an “immune evasion cluster”, or pathogenicity island (SaPln5), and is expressed early in the exponential phase (Rooijakkers et al., 2006). SCIN specifically binds and stabilises the C3 convertases C4b2a and C3bBb on activated surfaces and inhibits their ability to cleave C3 as determined by the inhibition of C3b deposition (Rooijakkers et al., 2005a).

iii) Inhibition of the complement cascade through C3b
Extracellular fibrinogen binding protein (Efb) is a 19 kDa secreted protein (Lee et al., 2004a). Efb is able to bind simultaneously to both fibronectin and C3. The C-terminal domain of Efb binds directly to the C3d portion of C3 with a dissociation constant (K_D) of approximately 0.24 μM (Lee et al., 2004b). Analysis of both the classical and alternative complement pathways by lysis of red blood cells, revealed that Efb is able to inhibit both pathways by >80%. It has been proposed that this is due to Efb-induced inhibition of C3b binding to activator surfaces (Lee et al., 2004b).

In addition to the effects described previously, Sak-activated plasminogen is also able to cleave C3b. Rooijakkers and colleagues (2005b) have shown that in the presence of plasminogen, endogenously produced Sak greatly diminishes the amount of C3b deposited on the surface of S. aureus when compared to a Sak-negative strain.

iv) Inhibition of chemotaxis by C5a
Complement C5a and formylated peptides released by bacteria induce chemotaxis of leukocytes to the site of inflammation (Cicchetti et al., 2002). Chemotaxis inhibitory protein (CHIPS) is a 14 kDa, secreted protein whose gene clusters with sak, efb and scin, and is found in >60% of S. aureus clinical isolates (de Haas et al., 2004). CHIPS binds directly to
both the C5a receptor and the formyl peptide receptor through distinct sites (C- and N-terminal sites, respectively) but appears unable to bind both simultaneously (Haas et al., 2004). By binding these receptors, CHIPS blocks signalling processes, which would normally activate chemotaxis of the leukocytes. The structure of CHIPS has been solved by NMR and reveals a β-grasp fold (Haas et al., 2005), described in more detail in section 1.5.3 iii.

1.4.2 Evasion of neutrophil killing by S. aureus

Even after internalisation by phagocytosis, S. aureus has a remarkable ability to survive and can even lyse neutrophils from the inside. An in-depth study into the gene regulation of S. aureus reveals major changes within 30 min post-phagocytosis, with a general upregulation of defensive proteins (Voyich et al., 2005). Discussed below are some of these proteins and the effects they have.

i) Factors that inhibit opsonophagocytosis of S. aureus

As mentioned previously, SpA and Sbi bind to the Fc portion of IgG (section 1.4.1 i), hence inhibiting recognition by Fcγ receptors which initiate phagocytosis. Similarly, Efb and Sak binding of the opsonin C3b is able to inhibit phagocytosis via recognition by Mac-1.

Capsule formation by S. aureus involves the coating of bacteria in a thin layer of polysaccharides, where type 5 and type 8 are the most common. Thakker and colleagues (1998) showed that optimal expression of capsular polysaccharide type 5 protected S. aureus from phagocytosis compared with capsule-defective mutant strains. They also showed this protection was reversible upon the addition of serum containing anti capsule type 5 antibodies. This indicated that the protective mechanism of the capsule was probably due to anti-opsonic effects.

ii) Factors that inhibit lysis of S. aureus in the phagolysosome

Yet another function of Sak is its ability to bind to and inactivate α-defensins, small cationic peptides with direct microbicidal effects that are released from primary granules (Jin et al., 2004). It does this by forming a 1:6 Sak:α-defensin stoichiometry complex. Interestingly sak is downregulated in S. aureus by up to 5-fold after phagocytosis (Voyich et al., 2005).
Marshall and Wilmoth (1981) identified 17 compounds responsible for the golden pigmentation of S. aureus. These were found to be triterpenoid carotenoids, with the major component (70-80%) being staphyloxanthin. Dehydrosqualene synthase (ctrM) and dehydrosqualene desaturase (ctrN) were found to be key enzymes involved in the production of staphyloxanthin (Wieland et al., 1994) and subsequent isogenic knockout of ctrM showed reduced pathogenicity and neutrophil survival by oxidative burst (Liu et al., 2005).

Modulation of surface glycans also plays a role in S. aureus survival. For example, proteins encoded by the dlt operon are involved in D-alanine esterification of surface-bound teichoic acids, a process which reduces the negative charge that attracts defensins (Peschel et al., 1999). Additionally, O-acetylation of muramic acid by O-acetyltransferase A (OatA) confers resistance to bacterial lysis by lysozyme, a muramidase that cleaves peptidoglycans between the glycosidic β1,4-linked residues of N-acetylmuramic acid and N-acetylglucosamine (Bera et al., 2005).

### iii) Toxins that lyse neutrophils

Expression of hemolysins and holins (proteins that lyse cells by forming holes in membranes) are greatly upregulated by S. aureus after phagocytosis by neutrophils (Voyich et al., 2005). In particular, γ-hemolysin is upregulated over 20-fold for up to and longer than 180 minutes after phagocytosis. Hemolysins, along with panton-valentine leukocidin (PVL), typically lyse leukocytes by forming β-barrel pores in the leukocyte membrane (Dinges et al., 2000). This results in the leakage of water and ions and cell lysis by osmotic shock.

### 1.4.3 Other evasion mechanisms

In order to hide from immune molecules, S. aureus is able to bind directly to endothelial cells and bacterial endocytosis is observed within 3 hours (Ogawa et al., 1985). This mechanism also serves to protect the bacteria from antibiotics. It also increases the production of small colony variants (SVC), which may have a role in tissue persistence (Vesga et al., 1996) due to decreased antibacterial susceptibility (Chuard et al., 1997). SVCs of S. aureus are slow-growing subpopulations which are phenotypically different from invasive strains. Culturing of SVCs however, often causes reversion to a wild-type phenotype (Sifri et al., 2006).
1.5 Virulence factors produced by *S. aureus*

In addition to host evasion molecules, *S. aureus* produces numerous exotoxins and virulence factors that cause morbidity and even mortality in infected populations. Examples of these virulence factors are described below.

1.5.1 Exfoliative toxins

Staphylococcal scalded skin syndrome (SSSS) is caused by the exfoliative toxins A and B (ETA, ETB). They are members of the trypsin-like serine proteases and share homology with the V8 protease, an enzyme that cleaves peptide bonds on the carbonyl side of Asp and Glu. (Plano, 2004). They have specificity for desmoglein-1, the predominant desmosomal cadherin in the upper epidermis of the skin (Amagai *et al.*, 2002). Alanine mutation of the catalytic triad conserved with other trypsin-like serine proteases did not affect the binding of ETB to desmoglein-1, but completely abolished its proteolytic activity (Hanakawa *et al.*, 2002). In effect, SSSS caused by ETB is essentially a bacteria-derived form of the autoimmune disease pemphigus foliaceus, where patients generate autoimmune antibodies against desmogleins (Amagai, 1999).

Yamaguchi and colleagues (2002a) identified a third exfoliative toxin, ETD which is associated with bullous impetigo (Yamaguchi *et al.*, 2002b). A fourth toxin, ETC, was identified from *S. aureus* isolated from horse, but has not been shown to have any association with human disease (Sato *et al.*, 1994).

1.5.2 Panton-valentine leukocidin

Although the gene encoding PVL is detected in less than 5% of clinical *S. aureus* strains, it no doubt has a role in pathogenesis. PVL has been implicated with numerous disease states and PVL-positive strains typically cause greater morbidity than PVL-negative strains (Yamasaki *et al.*, 2005). These include boils, folliculitis, sepsis and necrotising pneumonia (Iwatsuki *et al.*, 2006; Labandeira-Rey *et al.*, 2007). PVL can also cause neutrophil lysis as mentioned previously (section 1.4.2 iii).
1.5.3 Superantigens

The name “superantigen” (SAg) is given to a toxin able to over-stimulate the immune system in a non-specific manner (Marrack and Kappler, 1990). The best described SAgs belong to the gram-positive bacterial superfamily, which includes toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEA etc.), streptococcal pyrogenic exotoxins (SpeA etc.) and streptococcal mitogenic exotoxins (SMEZ-1 etc.). These proteins crosslink the major histocompatibility complex class II (MHC II) on antigen-presenting cells and the T-cell receptor (TcR) on T-lymphocytes, resulting in massive cell activation and cytokine release. Typically, peptide antigen-stimulated activation will stimulate one in 10^5-10^6 T-lymphocytes, whereas SAg-stimulation can activate up to one in five (Proft and Fraser, 2003).

The bacterial SAgs contain the conserved PROSITE (Hulo et al., 2006) motif K-x(2)-[LIVF]-x(4)-[LIVF]-D-x(3)-R-x(2)-L-x(5)-[LIV]-Y (entry PS00278). Sequence alignment of the SAgs with the related staphylococcal superantigen-like (SSL) proteins (see section 1.5.4) and the subsequent generation of a tree using the Neighbour-Joining (NJ) algorithm reveals clustering of related proteins (figure 1.5). Streptococcal superantigen (SSA), SpeA, SpeH and SpeI cluster with the Staphylococcal SAgs, indicating a possible lateral transfer event between the two species as the genes are located on mobile genetic elements. TSST-1 clusters at the bottom of the tree with the SSLs.

SAgs can also be produced by gram-negative bacteria e.g. Yersinia pseudotuberculosis (Yoshino et al., 1994) and viruses e.g. murine mammary tumor virus (MMTV) (Beutner et al., 1992), rabies virus (Lafon et al., 1992) and Epstein-Barr virus (Sutkowski et al., 1996).

To date, structures are only available for Y. pseudotuberculosis-derived mitogen A (YPMa), which forms a jelly roll (Donadini et al., 2004), and members of the superfamily from the gram-positive bacteria S. aureus and Streptococcus pyogenes. However, it is known that minor lymphocyte stimulating protein 1 (Mls1), produced by MMTV, is a type-II integral membrane protein with a small, intracellular N-terminal domain and a large, heavily glycosylated, extracellular C-terminal domain (Choi et al., 1992).

Figure 1.5 - Neighbour-Joining tree of SAgs and SSLs
Comparison of primary sequences of SAgs from S. pyogenes and S. aureus, and SSLs from S. aureus are presented as an N-J tree.
i) **Three-dimensional structure of classical bacterial SAgS**

The classic SAg fold, adopted by all SAgS produced by gram positive cocci, includes an N-terminal oligosaccharide / oligonucleotide binding domain (OB fold) and a C-terminal β-grasp domain (figure 1.6). Although amino acid sequence identity within this family is low, members maintain the conserved structural features, indicating a high tolerance for variation within these folds. The location of the MHC II and TcR binding sites are relatively conserved, with the MHC II binding site located either on the face of the β-grasp domain and incorporating a zinc atom, or centred on a hydrophobic ridge along the outer face of the OB-fold (Proft and Fraser, 2003). The TcR binding site is located in a shallow groove between the two domains and variation in this region accounts for the Vβ specificity (Papageorgiou and Acharya, 2000; Petersson *et al.*, 2004).
Figure 1.6 - The SAg fold

Ribbon diagrams of A) TSST-1 and B) SpeC generated from the pdb files 4TSS and 1AN8 respectively (Prasad et al., 1997; Roussel et al., 1997). The OB domain is coloured red, the β-grasp domain in blue and the N-terminal α-helix and domain linker in green. C) Schematic diagram of the prototypic secondary structure of the superantigen, SEB. Black arrows indicate β-strands and red bars indicate α-helices. The disulphide bond is present in some superantigens i.e. SEA, SEB, SEC and SSA.

ii) The OB fold

A search for OB folds in the Protein Data Bank (pdb) database reveals 101 examples originating from viral / phage, bacterial and eukaryotic origin, showing a steady increase from the 85 found in 2002 (Arcus, 2002). This fold was first discussed by Murzin (1993), who found a common fold between four oligonucleotide / oligosaccharide binding proteins (staphylococcal nuclease, the anticodon binding domain of asp-tRNA synthetase and the B-subunits of heat-labile enterotoxin and verotoxin-1). These proteins shared a consensus architecture originally described as a “β-barrel capped by an α-helix located between the third and fourth strands” (Murzin, 1993), although some members of this family lack the α-helix (Kloks et al., 2002). Arcus (2002) puts forward a more detailed description, as a five-stranded mixed β-barrel with a shear number of 8, or 10. The shear number, S, is a measure of stagger of the β-sheet (figure 1.7). OB folds share a common binding face which can recognize a range of ligands, due to the flexibility in amino acid sequence (Arcus, 2002).
iii) The β-grasp fold

The Structural Classification of Proteins (SCOP) (Murzin et al., 1995) lists 11 superfamilies under the β-grasp / ubiquitin-like fold, which include immunoglobulin-binding domains, staphylokinase / streptokinase, and the C-terminal domains of SAgs. The core structure is defined as β–β–α–β–β although a more general description is a mainly antiparallel β-sheet with a segregated α and β region. Similar to the OB fold, the β-grasp fold tends to have poor sequence identity among family members. There is variation in the packing and number of β-strands between proteins that share this fold (figure 1.8).

1.5.4 Staphylococcal Superantigen-Like proteins (SSLs)

A family of proteins that are structurally related to SAgs was recently described as staphylococcal exotoxin-like toxins (SETs). To remedy a previously confusing system in which alleles of the same protein were allocated different names, a standard nomenclature was proposed by Lina et al. (2004), in which SETs were renamed staphylococcal superantigen-like proteins (SSLs) and numbered in clockwise order from the origin of
replication. This naming system will be used throughout the remainder of this thesis. Like the SAgs, SSLs also contain the conserved PROSITE motif K-x(2)-[LIVF]-x(4)-[LIVF]-D-x(3)-R-x(2)-L-x(5)-[LIV]-Y (Hulo et al., 2006). However, the literature suggests that the SSLs have functions that are distinct from the SAgs. The reported expression, functions and structures of the SSLs are described below.

i) Genetics of the ssls
A novel cluster of at least five genes encoding SSLs was first published by Williams and colleagues (2000). Shortly thereafter, the publication of complete S. aureus genomes revealed that there were, in fact, a cluster of up to 11 related genes within a "pathogenicity island" (figure 1.9) (Baba et al., 2002; Kuroda et al., 2001). This island also contains a cluster of lipoprotein-like proteins of unknown function and hsdM and hsdS genes, which are part of the type-IC restriction modification system. Three other genes encoding SSLs (ssl12-14) are located further upstream in what has been called an immune evasion cluster which also contains genes encoding SCIN, Efb, and CHIPS (see sections 1.4.1 ii, iii and iv)

Restriction modification systems perform three main roles; first to attack “non-self” DNA i.e. phages, second to kill cells that no longer maintain them, and third to enable genetic transfer (reviewed by Kobayashi, 2001). Genetic transfer of a related pathogenicity island of S. aureus, containing the gene encoding TSST-1, has been shown to occur (Lindsay et al., 1998).
The *ssl* genes are present in all *S. aureus* isolates tested, from human, bovine (cow), caprine (goat), ovine (sheep), leporine (rabbit) and avian (chicken) sources, although some strains do not contain the full complement of the *ssl* genes (Fitzgerald *et al*., 2003; Smyth *et al*., 2007). An evolutionary model proposed by Fitzgerald *et al*. (2003) suggests that an *S. aureus* ancestor originally contained a full complement of *ssl* genes, which have been selectively lost over time (figure 1.10). Aguiar-Alves and colleagues (2006) have proposed utilising the variation in presence and sequence of *ssl* genes as a genotyping tool, which they have shown is able to distinguish MRSA and MSSA isolates in more defined subgroups than the commonly used multilocus sequence typing.
Figure 1.10 - Evolution of the ssl genes in pathogenicity island 2

Proposed model by which extant distributions of ssl genes within the second pathogenicity island have formed, modified from Fitzgerald et al. (2003). Isolates of S. aureus are named down the right side of the tree created using the Neighbour-Joining (NJ) algorithm, with gene loss indicated in red. Bootstrap confidence limits are indicated at the major nodes.

ii) Expression of SSLs

Coordinated expression of unrelated proteins by S. aureus is controlled by global gene regulators. These fall into two categories: the two-component regulatory systems (TCRS), of which S. aureus has 16, and the SarA homologues (Cheung et al., 2004). The expression of these regulators is temporal, i.e. SarA expression peaks during late-exponential phase, while the TCRS agr is more prominent in post-exponential phase (Cheung et al., 2004). Expression of the SAgs, hemolysins and proteolytic enzymes are controlled by agr, while expression of some surface proteins such as SpA and fibrinogen-binding proteins are controlled by SarA homologues (Arvidson and Tegmark, 2001; Novick, 2003). The expression of the ssl genes appears to be tightly regulated (Fitzgerald et al., 2003) and responsive to the host environment. For example, SSLs 2, 3 and 5 are upregulated in S. aureus over a period of 180 minutes, after phagocytosis by neutrophils (Voyich et al., 2005).
Reactive antibodies against SSLs 5, 7 and 9 were found in most donors and production and secretion of SSL5 by *S. aureus* is evident by western analysis (Al-Shangiti *et al.*, 2005; Arcus *et al.*, 2002). Fitzgerald and colleagues (2003) found reactive antibodies against SSLs 2, 3, 4, 7, 8 and 10 in patients admitted with invasive *S. aureus* infections. Expression of the *ssl7* gene is evident at early to mid log phase, but is greatly increased at late log and stationary phase (Williams *et al.*, 2000). Similarly, *ssl1*-4 are expressed in lower levels at mid-log phase but are greatly upregulated during stationary growth (Fitzgerald *et al.*, 2003).

### iii) Analysis of SSL function

The investigation into the functions of SSLs is relatively recent and as a result, there have been few publications to date. In the first report by Williams and colleagues (2000), they suggested that SET1 (now SSL7) was able to stimulate proinflammatory cytokine production. However, it has since been shown that it is unable to stimulate peripheral blood lymphocyte proliferation (Arcus *et al.*, 2002) or induce fever in rabbits (Fitzgerald *et al.*, 2003), suggesting a function that is distinct from SAgS.

SSL7 exhibits highly specific, simultaneous binding to both the complement component C5 and IgA with dissociation constant (K_D) values in the nanomolar range. Functionally, SSL7 is able to inhibit complement-mediated cell lysis via the alternative pathway. Binding of SSL7 to IgA occurs at the Ca2/Ca3 interface (Wines *et al.*, 2006) and competes for binding to the myeloid receptor, Fc_αRI (CD89) (Langley *et al.*, 2005) which, when activated, can elicit phagocytosis, degranulation and respiratory burst (Monteiro and Van De Winkel, 2003). The crystal structure of an SSL7-IgA complex has recently been solved and confirms this (Wines *et al.* unpublished). SSL7 along with SSL9 have shown to internalize into small vesicular structures in dendritic cells (Al-Shangiti *et al.*, 2004).

SSL5 binds to the P-selectin glycoprotein ligand-1 (PSGL-1) with an apparent K_D of 820 ± 540 nM (Bestebroer *et al.*, 2006). PSGL-1 binds its native ligand, P-selectin, with a K_D of 340 ± 20 nM (Mehta *et al.*, 1998). SSL5 competes with P-selectin in a dose-dependent manner and inhibits the tethering of neutrophils under shear conditions, where a 72% reduction of adherent cells was observed after incubation with 4 μM SSL5 (Bestebroer *et al.*, 2006). Similar to the interaction with SSL5, P-selectin requires the sulphation (Wilkins *et al.*, 2004).
1995) and sialation (Moore et al., 1992) of PSGL-1 for high-affinity binding, indicative of target specificity.

iv) Analysis of SSL structures

The two best characterized SSLs are SSL5 (previously SET3) and SSL7, for which crystal structures have been solved at 1.9 Å and 2.7 Å resolution respectively (Al-Shangiti et al., 2004; Arcus et al., 2002). When the two structures are superimposed by secondary structure matching (SSM), 175 residues can be matched with a root mean square difference (rmsd) of 1.69 Å in Cα positions, indicating a high structural conservation (figure 1.11A). Both proteins form proposed crystallographic dimers by interaction across the β-grasp domain, although they are present as monomers in solution. The SSL5 dimer has a much smaller buried surface area (287 Å²/monomer – figure 1.11B) compared to that of SSL7 (1122 Å² and 1146 Å² for the two crystal forms solved – figure 1.11C). SSL5 has a calculated pI of 9.4 and SSL7 has a calculated pI of 7.2. This is apparent in the structures as SSL5 has large patches of high positive surface charge, particularly in the saddle-shaped region formed at the site of dimerisation (figure 1.11B). It was suggested that negatively-charged DNA could bind within the saddle-shaped region (Arcus et al., 2002). Conversely, SSL7 has a strip of highly negative surface charge at the region of dimerisation (figure 1.11C).

Although SAgs and SSLs share a highly conserved architecture, there are some notable differences between them. Typically, SAgs have an extended loop between β2 and β3 when compared with SSL5 and SSL7. The exceptions are SEH and TSST-1, both of which are outliers from the main groups of SAgs (figure 1.5). The SAgs also have extended loops between the β-strands β4 and β5 in the OB domain, and the β-strands β10 and β11 in the β-grasp domain. Both SSL5 and SSL7 exhibit an elongation of β-strands β6 and β7 compared to SAgs, resulting in a prominent β6-β7 hairpin loop.
v) Staphylococcal Superantigen-like protein 11 (SSL11)

SSL11 is expressed in the late-exponential / stationary phase, with peak expression between 30 and 40 h in 50% BHI medium, by a SarA P3 and agr-independent mechanism (Laughton et al., 2006). Rather, SSL11 appears to be tightly regulated by the TCRS, SaeRS, which comprises the response regulator SaeR and the histidine kinase SaeS (Rogasch et al., 2006). Interestingly, other SSLs are not similarly regulated. SaeRS expression is negatively regulated by 1M NaCl, pH <6.0, and subinhibitory concentrations of the lincosamide antibiotic, clindamycin (Novick and Jiang, 2003). In contrast, SaeRS is upregulated by the β-lactam antibiotics, vancomycin and cefoxitin (Kuroda et al., 2007; Kuroda et al., 2003).
Distinct differences in SSL11 expression were observed between the COL and Newman strains of *S. aureus* due to comparably higher expression of *SaeS* in *S. aureus* Newman (Rogasch et al., 2006). One possible reason for the differential expression is that COL is a meticillin-resistant strain (Shafer and Iandolo, 1979), while Newman is an meticillin-sensitive strain (Dajcs et al., 2002). An isogenic knockout of *SaeS* in *S. aureus* Newman resulted in a significant reduction in expression of SSL11 (Rogasch et al., 2006). In fact, the gene that *SaeS* had the greatest effect on expression of, was *ssl11* (referred to as *set15*), with a 75-fold difference observed between a wild-type strain (RN4220) and a *SaeS* null mutant (Liang et al., 2006). The *SaeS* null mutant had significantly reduced adherence to, and internalization into endothelial cells, significantly reduced killing of endothelial cells, and significantly attenuated virulence in a mouse model compared to the wild-type strain. These observations correlate with similar results in a post-surgery rat model, where virulence was attenuated by a soluble factor produced by *Lactobacillus reuteri* RC-14 that also significantly reduced the expression of SSL11 (Gan et al., 2002; Laughton et al., 2006). In addition, a recent study has shown that *S. aureus* endocytosed into endothelial cells upregulates expression of *ssl11* by ~5-fold within 6 h (Garzoni et al., 2007). These studies implicate SSL11 as having an important role in the pathogenicity of *S. aureus*.

### 1.6 Aims

Research performed by Dr. Ries Langley (Langley, 2003) suggested that SSL11 may have an important function in *S. aureus* virulence involving the IgA receptor, FcαRI. However, little else is known about SSL11. Therefore, this research aimed to characterise the structure and function of SSL11, including its interaction with FcαRI and other putative binding partners.
Chapter 2 - Methods and Materials

2.1 Materials

2.1.1 DNA analysis

i) Common buffers

<table>
<thead>
<tr>
<th>Buffer/Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>50 mM Tris-HCl pH 7.4, 150 mM NaCl, 20 mM EDTA, 5 μg/mL lysostaphin, 50 μg/mL DNAse-free RNase A, 0.5 mg/mL proteinase K, 0.5% w/v SDS</td>
</tr>
<tr>
<td>DNA loading buffer</td>
<td>0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 15% v/v Ficoll (type 400; Pharmacia)</td>
</tr>
<tr>
<td>Solution I</td>
<td>25 mM Tris-HCl pH 8.0, 50 mM Glucose, 10 mM EDTA</td>
</tr>
<tr>
<td>Solution II</td>
<td>0.2 M NaOH, 1% w/v SDS</td>
</tr>
<tr>
<td>Solution III</td>
<td>3M KOAc, 11.5% v/v glacial acetic acid</td>
</tr>
<tr>
<td>TAE</td>
<td>40 mM Tris acetate, 2 mM EDTA</td>
</tr>
<tr>
<td>TE buffer</td>
<td>10 mM Tris-HCl pH 7.5, 1 mM EDTA</td>
</tr>
<tr>
<td>TFB I</td>
<td>100 mM RbCl, 10 mM CaCl₂, 50 mM MgCl₂, 30 mM KOAc, 15% glycerol, adjusted to pH 5.8 with acetic acid</td>
</tr>
<tr>
<td>TFB II</td>
<td>10 mM MOPS pH 7.0, 10 mM RbCl, 75 mM CaCl₂, 15% glycerol</td>
</tr>
</tbody>
</table>

ii) Oligonucleotides

Primers were purchased from Sigma Genosys (see appendix 7.1).

iii) Plasmids

See appendix 7.2 for plasmid details.

- pBluescript: Ampicillin-resistant vector from Stratagene enabling blue / white selection
- pBC: Chloramphenicol-resistant version of pBluescript (Stratagene)
- pET32a3C: pET32a vector (Novagen) modified to contain a 3C protease site 3′ of the thioredoxin gene, trxA.
pGEX3C pGEX-2T vector (Pharmacia) modified to contain a 3C protease site 3’ of the glutathione s-transferase gene, gst.

iv) Bacterial strains

*E. coli* DH5α (ATCC) and AD494(DE3)pLysS (Novagen).

*S. aureus* Clinical isolates were obtained from Greenlane Hospital, New Zealand (denoted with NU), Auckland Hospital, New Zealand (denoted with US) or various hospitals in Germany (see appendix 7.3).

2.1.2 Protein analysis

i) Antibodies

Mouse anti-human-CD10 and CD89 (clone MIP8a) monoclonal antibodies (mAb) were purchased from Serotec as phycoerythrin (PE) conjugates or unconjugated. Mouse anti-human-CD89 (clone A59) mAb were purchased from BD Biosciences as PE conjugates. Mouse anti-human-sLe^x^ (clone KM93) mAb was purchased from Calbiochem. Goat anti-mouse, -human and -rabbit antibodies conjugated to fluorescein isothiocyanate (FITC) or horseradish peroxidase (HRP) were purchased from Serotec or Dako Cytomation.

ii) Buffers and solutions

Anhydrous acetone Molecular sieve added at 16 g/L to acetone for at least 12 h

Coomassie Blue stain 50% v/v ethanol, 7.5% v/v glacial acetic acid, 0.05% w/v Coomassie Brilliant Blue R250

Ponceau S stain 0.1% w/v Ponceau S, 5% v/v glacial acetic acid.

2x Loading buffer 100 mM Tris-HCl pH 6.8, 4.0% w/v SDS, 0.2% w/v bromophenol blue, 20% v/v glycerol

Laemmli buffer 25 mM Tris-HCl pH 8.8, 192 mM glycine, 0.1% w/v SDS

Towbin’s buffer 25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.375% w/v SDS, 20% v/v methanol

PBS 120 mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄ pH 7.4

PBS-T PBS with 0.05% v/v Tween 20

PBS-BSA PBS with 0.5% w/v bovine serum albumin fraction V

TBS 10 mM Tris-HCl pH 8.0, 120 mM NaCl
2.5% Blotto  TBS with 2.5% w/v non-fat dairy milk powder
5% Blotto  TBS with 5% w/v non-fat dairy milk powder
TBS-T  TBS with 0.05% v/v Tween 20
TSA  10 mM Tris-HCl pH 8.0, 140 mM NaCl, 0.025% w/v NaN₃
Wash buffer  10 mM Tris-HCl pH 8.0, 500 mM NaCl, 1% v/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v SDS
GSH buffer I  25 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM EDTA
GSH buffer II  25 mM Tris-HCl pH 7.4, 0.5 M NaCl, 1 mM EDTA
NTA buffer I  50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% v/v glycerol
NTA buffer II  50 mM sodium phosphate pH 8.0, 300 mM NaCl, 100 mM imidazole
NTA buffer III  50 mM sodium phosphate pH 8.0, 300 mM NaCl
HBS-EP  10 mM HEPES-HCl pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% v/v P20 surfactant
RBC lysis buffer  10 mM KHCO₃, 168 mM NH₄Cl, 0.13 mM EDTA pH 7.4
Cell lysis buffer  50 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.5% v/v Triton X-100, 1 tablet of mini EDTA-free protease inhibitor cocktail (Roche) per 10 mL.

iii) Media
Luria Bertani (LB) broth  1% w/v Bacto tryptone, 0.5% w/v Bacto yeast extract, 1% w/v NaCl
LB agar plates  LB broth containing 1.5% Bacto agar
Terrific Broth  1.18% w/v Peptone, 2.36% w/v Bacto yeast extract, 54 mM K₂HPO₄, 16.2 mM KH₂PO₄, 0.4% v/v glycerol

2.1.3 Cell analysis

i) Cell lines
266CHO K1 D8.10  Chinese hamster ovary cell line that was stably transfected with pBAR266 (see appendix 7.2) to constitutively express and secrete recombinant FcαRI.
ii) Cell culture media
All media was supplemented with 50 μg/mL geneticin (G418) to maintain the transfected plasmid (pBAR266).

| Complete RPMI 5% FCS | Roswell Park Memorial Institute 1640 media supplemented with 5% heat inactivated FCS |
| EX-CELL™ 325 PF CHO | Protein and serum free media specially designed for the cultivation of CHO cells (JRH Biosciences) |

2.2 Methods

2.2.1 DNA analysis

i) Purification of genomic DNA from S. aureus
Liquid cultures of S. aureus were grown overnight in 10 mL LB broth at 37 °C on a shaker (200 rpm). Bacteria were recovered by centrifugation (4,000 g for 5 min), resuspended in 0.5 mL of lysis buffer and incubated at 37 °C for 30 min. The bacterial lysate was incubated with 0.5 mL phenol with constant mixing by inversion at 4 °C before centrifugation at 16,000 g for 10 min. The top layer containing the genomic DNA was transferred to a new tube and an equal volume of 1:1 phenol:chloroform added. The contents were mixed by gentle inversion and centrifuged at 16,000 g for 10 min. The phenol:chloroform extraction was repeated once. DNA was precipitated by ethanol as follows. The top layer was transferred to a new tube, 0.1x volume of 3 M NaOAc pH 5.4 and 2x volumes of pure ethanol were added and mixed by gentle inversion. The mixture was allowed to stand at RT for 5 min before centrifugation at 16,000 g for 10 min. The supernatant was discarded and remaining traces of ethanol in the DNA pellet were evaporated at RT for 10 min. DNA was resuspended in 50 μL sterile, autoclaved milliQ H₂O.

ii) Purification of genomic DNA from E. coli
E. coli strain DH5α was cultured overnight in 5 mL LB broth at 37 °C with shaking (200 rpm). Bacteria from a 1.5 mL aliquot were harvested by centrifugation at 5,000 g for 2 min, resuspended in 600 μL TE buffer with 0.5% w/v SDS and 100 μg/mL proteinase K then incubated for 1 h at 37 °C. The bacterial lysate was mixed with 100 μL of 5 M NaCl and 80
μL of 10% CTAB (hexadecyltrimethyl ammonium bromide) in 0.7 M NaCl and incubated for 10 min at 65 °C. Lipids were removed by chloroform extraction as follows. An equal volume of chloroform was added to the lysate (780 μL), mixed by inversion and centrifuged at 16,000 g for 10 min. The top layer containing the genomic DNA was transferred to a fresh tube and cleaned with a phenol:chloroform extraction (see section 2.2.1 ii). The top layer was transferred to a new tube and the genomic DNA precipitated by addition of 0.6 mL isopropanol. The tube was centrifuged at 16,000 g for 10 min and the DNA pellet washed with 70% ethanol. The supernatant was removed and the pellet resuspended in 50 μL TE buffer.

iii) Plasmid preparation by alkaline lysis
Liquid cultures of *E. coli* transformed with the plasmid of interest were grown overnight in LB broth containing the appropriate antibiotics at 37 °C with shaking (200 rpm). Generally 3-5 mL were grown for small scale and 20 mL for large scale (stock) preparations. The method described is for small scale preparation of plasmid DNA, but can be upscaled using appropriate volumes. Bacteria were recovered by centrifugation (4,000 g for 5 min) and resuspended in 100 μL of solution I by pipetting. Bacteria were lysed by the addition of 200 μL solution II and mixing by gentle inversion. The solution was neutralised by the addition of 150 μL solution III, followed by mixing by gentle inversion. Bacterial debris was removed by centrifugation at 16,000 g for 10 min. The supernatant was transferred to a new tube and 450 μL of pure ethanol added and incubated at RT for 10 min. The tube was centrifuged at 16,000 g for 10 min. The pellet containing the plasmid DNA was resuspended in 200 μL of sterile milliQ H2O containing 10 μg/mL DNAse-free RNAse A and incubated at 50 °C for 30 min. Plasmid DNA was then cleaned by phenol:chloroform extraction and ethanol precipitation (see section 2.2.1 ii) and resuspended in 50 μL sterile milliQ H2O.

iv) Plasmid preparation for sequencing
A 45 μL aliquot of the plasmid preparation, described in section 2.2.1 iii, was mixed with 5 μL 2 M NaOH, 2 mM EDTA and incubated at 37 °C for 30 min. To this mix, 75 μL sterile milliQ H2O, 7 μL of 10 mg/mL ethidium bromide and 70 μL of 7.5 M NH4OAc were added and mixed by inversion. A phenol:chloroform extraction was performed (see section 2.2.1 ii), but with mixing by vortexing. The top layer was transferred to a new tube and the plasmid DNA precipitated by the addition of 400 μL of pure ethanol and incubation at RT for 2 min.
The tube was centrifuged at 16,000 \( g \) for 10 min, the supernatant discarded and remaining traces of ethanol in the DNA pellet were evaporated at RT for 10 min. Plasmid DNA was resuspended in 10 \( \mu \)L autoclaved, sterile milliQ \( H_2O \), quantified by UV spectroscopy and sequenced either on a LICOR 4000LD IR2 at the Faculty of Medical Health Sciences DNA Sequencing Facility, University of Auckland, or a capillary ABI3730 Genetic Analyzer (Applied Biosystems Inc.) at the Alan Wilson Centre, Massey University, Auckland.

v) Agarose gel electrophoresis

DNA loading buffer was added to DNA at a working concentration of 1x and samples were electrophoresed at 100 V through 1% w/v agarose in TAE. The gel was stained by slow shaking (45 rpm) in TAE buffer with \( \sim 5 \mu g/mL \) ethidium bromide for 10 min at RT, rinsed briefly with deionised \( H_2O \) and visualised using a Gel Doc 2000 (Bio-Rad).

vi) Restriction endonuclease digestion of DNA

Typically, DNA was digested in a final volume of 100 \( \mu \)L with 1x appropriate reaction buffer (supplied by manufacturer) and 0.5-1 \( \mu \)L of enzyme at 37 °C for at least 30 min. In some instances, \( \sim \)5 units of calf intestinal alkaline phosphatase (CIP) were added to either linearised plasmid or insert (but not both) for a further 30 min at 37 °C to reduce self-ligation. DNA was cleaned by phenol:chloroform extraction and ethanol precipitation (see section 2.2.1 ii).

vii) Preparation of T-tailed plasmid

One microgram of pBluescript or pBC was linearised with 5 units of \( EcoRV \) in a total volume of 10 \( \mu \)L at 37 °C for 30 min. The reaction was made up to a final volume of 50 \( \mu \)L containing 1x Taq buffer, 2.5 mM MgCl2, 200 nM dTTP and 5 units of Taq polymerase and incubated at 72 °C for 30 min. The reaction was transferred to a microfuge tube and 75 \( \mu \)L of 7M NH4OAc and 75 \( \mu \)L sterile milliQ \( H_2O \) were added. T-tailed DNA was cleaned by phenol:chloroform extraction followed with precipitation by addition of 2.5x volumes of pure ethanol. The DNA was resuspended in 10 \( \mu \)L of autoclaved, sterile milliQ \( H_2O \) for future use.

viii) Polymerase Chain Reaction (PCR)

PCR was typically performed in a total volume of 50 \( \mu \)L containing 2.5 units of Taq polymerase, 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl), 2.5 mM MgCl2, 100 \( \mu M \)
of each dNTP, 0.1 μM of primer and either bacteria from a colony for screening purposes, or purified DNA as template. Reactions were run in a PX2 thermal cycler (Medica Pacifica Ltd) with the following cycles: denaturation at 94 °C for 2 min, followed by repeated cycles of [94 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s] and a single cycle at 72 °C for 5 min. For amplification from genomic DNA, 30 cycles were used and for amplification from plasmid DNA, 15 cycles were typically used.

**ix) Purification of DNA from agarose gel**

DNA bands were excised from the agarose gel over a UV source, where caution was used to have minimal exposure time to reduce DNA damage. The gel blocks were transferred to separate microfuge tubes and frozen at -80 °C. The gel blocks were then thawed, briefly centrifuged and “smashed” by physical grinding. The gel was again frozen and thawed, then centrifuged at 16,000 g for 2 min. The liquid containing the DNA was transferred to a new tube by pipette for further use.

**x) Ligation of DNA**

Purified plasmid and insert DNA containing compatible ends were mixed at a molar ratio of between 1:3 and 1:5 and ligated in a final volume of 10 μL using QuickStick Ligase (Bioline) in the supplied buffer for at least 10 min at RT.

**xi) Site-directed mutagenesis by overlap PCR**

Site-directed mutants of SSL11 were generated by overlap PCR. In the first step, “forward” and “reverse” fragments were amplified from plasmid DNA by 15 cycles of PCR, using the primer pairs SSL11-N315-Fw, T168P-Rv and SSL11-N315-Rv, T168P-Fw (see appendix 7.1). The fragments were separated from template DNA by agarose gel electrophoresis, excision and gel purification. In the second step, the fragments were mixed in a 1:1 molar ratio and full length product was amplified using the primers SSL11-N315-Fw and SSL11-N315-Rv by 15 cycles of PCR. The amplified DNA was then digested with *BamHI* and *EcoRI* and electrophoresed through an agarose gel. The full length product was excised, purified and ligated into pGEX3C similarly digested with *BamHI* and *EcoRI*. 
xii) Production of chemically competent *E. coli*

A 5 mL starter culture of *E. coli* was grown overnight in LB broth containing 20 mM MgSO\(_4\) and 10 mM KCl at 37 °C with shaking (200 rpm). The next morning, 1 mL of the starter culture was used to inoculate 100 mL of LB broth containing 20 mM MgSO\(_4\) and 10 mM KCl and allowed to continue growing at 37 °C with shaking (200 rpm). Bacteria were harvested by centrifugation at 5,000 g for 5 min at 4 °C when an optical density of 0.3-0.4 at 600 nm was reached. The bacterial pellet was gently resuspended in 60 mL of ice-cold TFB I and incubated on ice for 10 min. Bacteria were centrifuged at 5,000 g for 5 min at 4 °C gently resuspended in 4 mL ice-cold TFB II, aliquoted, flash frozen in a dry ice / ethanol bath and stored at -80 °C for future use. All steps were performed in sterile conditions.

xiii) Transformation of chemically competent *E. coli*

Half of the ligation mix from section 2.2.1 x (5 μL) was added to 50 μL of chemically competent *E. coli* and incubated on ice for 5 min. The bacteria were heat shocked at 42 °C for 45 s followed by incubation on ice for 5 min. LB broth was added to a final volume of 500 μL and the bacteria incubated at 37 °C for 30 min. The bacteria were recovered by centrifugation at 5,000 g for 5 min, resuspended in 100 μL of LB broth and for blue/white selection, 4 μL 1 M IPTG and 40 μL 2% w/v X-gal were added. The bacteria were plated on LB-agar plates containing the appropriate antibiotics and incubated overnight at 37 °C.

2.2.2 Protein analysis

i) Production of IDA and GSH sepharose

Sepharose 4B-CL (100 mL) was thoroughly washed with deionised water through a sintered glass filter and suctioned dry. The gel was resuspended in 100 mL 35% w/v diglycidyl ether, 0.3 M NaOH, 1 mg/mL NaBH\(_4\) and rotated overnight at RT in a well-sealed bottle. The gel was washed thoroughly with deionised water through a sintered glass filter and suctioned dry. The sepharose was resuspended in 100 mL 50 mM NaPO\(_4\) with 1 g iminodiacetic acid (IDA) or reduced glutathione (GSH), adjusted to pH 8.0. Nitrogen was bubbled through the solution before incubation overnight at 37 °C with constant mixing by rotation. The gel was washed thoroughly with deionised water through a sintered glass filter. IDA sepharose was charged with nickel by passing through 100 mM NiSO\(_4\). The beads were tested for binding capacity (typically 6-10 mg fusion protein / mL beads) and stored in 20% ethanol at 4 °C.
ii) Protein expression and purification from *E. coli*

*E. coli* previously transformed with expression vectors (pGEX3C or pET32a3C containing the gene of interest) were cultured overnight at 37 °C with shaking (200 rpm) in 100 mL LB broth containing the appropriate antibiotics (*i.e.* cultures of DH5α transformed with pGEX3C were supplemented with 50 μg/mL ampicillin while cultures of AD494(DE3)pLysS transformed with pET32a3C were supplemented with 50 μg/mL ampicillin, 34 μg/mL chloramphenicol and 15 μg/mL kanamycin). The next morning, the overnight culture was transferred to 900 mL LB broth (total volume of 1 L) supplemented with the appropriate antibiotics and allowed to continue growing for another hour at 37 °C with shaking (200 rpm). The culture was then cooled on ice for 5 min, IPTG was added to 0.1 mM and the culture was allowed to continue growing for 3-4 h at 28 °C with shaking. Bacteria were harvested by centrifugation at 5,000 g for 10 min at 4 °C, the supernatant discarded and the pellet frozen at -80 °C. The bacterial pellet was then processed for the two systems as described below.

iii) Using the pGEX3C expression system

The bacterial pellet was resuspended in 50 mL GSH buffer I containing 1% v/v Triton X-100 and 0.1 mM PMSF and allowed to cool on ice. The bacteria were lysed by sonication using a Misonix XL2015 sonicator over 2x 1 min at high power with a 75% pulse. The insoluble fraction and bacterial debris were removed by centrifugation at 16,000 g for 10 min. The supernatant containing the soluble proteins was passed through a GSH sepharose column equilibrated with GSH buffer I. The column was washed with at least 10 column volumes (cv) of GSH buffer II and 5 cv of GSH buffer I. The beads were resuspended in GSH buffer I containing 1 mM dithiothreitol (DTT) and 3C protease at 10 μg per mg of estimated fusion protein (typically 10-30 mg/L of liquid culture) and incubated overnight at 4 °C with constant mixing by inversion. Protein was eluted by passing 1 cv of GSH buffer I through the beads and the purity checked by SDS-PAGE (section 2.2.2 viii).

Alternatively, after washing the column with 5 cv of GSH buffer I, bound protein was eluted in GSH buffer I containing 5 mM reduced GSH. Fusion protein was cleaved by addition of 1 mM DTT and 3C protease as above, and incubation overnight at 4 °C. GST was separated from the desired recombinant protein by anion exchange chromatography (section 2.2.2 vii). Typically, protein was further purified by size exclusion chromatography (section 2.2.2 vi).
iv) **Using the pET32a3C expression system**

The bacterial pellet was resuspended in 50 mL NTA buffer I containing 1% v/v Triton X-100 and 0.1 mM PMSF and allowed to cool on ice. The bacteria were lysed by sonication using a Misonix XL2015 sonicator over 2x 1 min at high power with a 75% pulse. The insoluble fraction and bacterial debris were removed by centrifugation at 16,000 g for 10 min. The supernatant containing the soluble proteins was passed over an IDA sepharose column pre-charged with 100 mM NiSO₄ and equilibrated with NTA buffer I. The column was washed with at least 10 cv of NTA buffer I followed by 5 cv of NTA buffer III. The beads were resuspended in NTA buffer III containing 1 mM DTT and 3C protease at 10 μg per mg of estimated fusion protein (typically 10-30 mg/L of liquid culture) and incubated overnight at 4 °C with constant mixing by inversion. Protein was eluted by passing 1 cv of NTA buffer III through the beads and the purity checked by SDS-PAGE (section 2.2.2 viii). Typically, protein was further purified by size exclusion chromatography (section 2.2.2 vi).

Alternatively, after washing with 10 cv of NTA buffer III, bound proteins were eluted in NTA buffer II. DTT was added to 1 mM and 3C protease added as above, to fractions containing protein. The fractions were incubated at 4 °C overnight, dialysed into NTA buffer III and passed back through IDA sepharose beads to remove the thioredoxin, before size exclusion chromatography (section 2.2.2 vi). In general, the second method resulted in higher yields of purified protein.

v) **Large scale preparation of recombinant protein expressed by E. coli**

A 500 mL starter culture of Terrific broth containing the appropriate antibiotics and inoculated with the *E. coli* strain of interest was grown overnight at 37 °C with shaking (200 rpm). The next morning, the culture was transferred to 4.5 L of Terrific broth containing the appropriate antibiotics in a Bioflo 3000 (New Brunswick Scientific) and grown for an hour at 28 °C with constant mixing and aeration. IPTG was added to 0.1 mM and the culture was grown for a further 6-7 h at 28 °C with constant mixing and aeration. Foaming was inhibited by the addition of 1 mL Antifoam (Sigma). The bacteria were harvested by centrifugation at 6000 g for 10 min, resuspended in 250 mL of either GSH buffer I or NTA buffer 1 as appropriate and frozen at -80 °C. The bacteria were thawed and refrozen at -80 °C twice more. Lysozyme was added to 0.2 mg/mL and the bacteria incubated at 50 °C for 30 min. The bacterial suspension was then cooled on ice and sonicated for 2x 1 min at high power.
Insoluble protein and bacterial debris were removed by centrifugation at 16,000 g for 15 min and the supernatant further processed over a GSH or IDA column as applicable.

vi) **Size exclusion chromatography**
Recombinant protein was typically concentrated using a Vivaspin (Sartorius) centrifugal concentrator prior to size exclusion chromatography, using a Superdex 200 10/300 GL column (Amersham) connected to an ÄKTA fast protein liquid chromatography (FPLC) system (Amersham). The column was equilibrated in the desired final buffer (*i.e.* PBS or 20 mM Tris-HCl pH 7.4) and a maximum of 0.5 mL protein was loaded and perfused at a flow rate of up to 0.5 mL/min. Fractions were collected by peak absorbance at 280 nm.

vii) **Anion exchange chromatography**
Recombinant protein was buffer-exchanged into 20 mM Tris-HCl pH 8.4 by dialysis and loaded onto a Poros 20HQ column (Perseptive Biosystems) connected to an ÄKTA FPLC system (Amersham). Bound protein was eluted by performing a salt gradient from 20 mM Tris-HCl pH 8.4 to 20 mM Tris pH 8.4, 500 mM NaCl.

viii) **Protein separation by SDS polyacrylamide gel electrophoresis (PAGE)**
Proteins samples were diluted 1:1 with 2x loading buffer, with or without 300 nM β-mercaptoethanol for reducing or non-reducing gels respectively and denatured at 94 °C for 2 min. Samples were electrophoresed at 200 V at a current of 20 mA per gel in Laemmli buffer, using Mighty Small II (Hoefer) apparatus. Typically, 12.5% acrylamide running gels with a 4% acrylamide stacker (Sambrook *et al.*, 1989) were used.

ix) **Coomassie Blue staining of proteins**
After polyacrylamide gel electrophoresis, proteins were stained in Coomassie Blue stain for at least 30 min at RT with slow shaking (~45 rpm). The gel was then rinsed with deionised H₂O and destained in 25% v/v ethanol, 8% v/v glacial acetic acid, using tissue paper as a wick to absorb excess dye.

x) **Silver staining of proteins**
For an increased level of detection, proteins were stained with silver after Coomassie staining. After the gel was destained, it was incubated for 5 min in H₂O then fixed in 13.5%
formaldehyde, 40% v/v methanol for 10 min. The gel was washed 2x for 10 min in H2O, then incubated in 0.02% Na2S2O3 for 1 min, followed by rinsing with H2O for 20 s. The gel was then incubated in 0.1% w/v AgNO3 for 10 min and rinsed with H2O. Staining was developed by incubating the gel in 3% Na2CO3, 0.05% formaldehyde, 0.0004% Na2S2O3 until bands started to become visible, then an equal volume of 2.3 M acetic acid was added to quench the reaction.

xi) Preparation of protein samples for identification by MALDI-MS
Protein samples were separated by SDS-PAGE and stained with fresh Coomassie Blue stain as above. The gel was destained in 25% v/v ethanol, 8% v/v glacial acetic acid without a wick. Bands were excised, cut into 1 mm3 pieces and stored at 4 °C. Proteins were identified at the Australian Proteome Analysis Facility (APAF) Ltd by MALDI-MS.

xii) Western analysis of proteins
Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane using a TE77 semi-dry transfer unit (Hoefer) with Towbin’s buffer. The membrane was reversibly stained with Ponceau S stain to check for efficient protein transfer and destained by washing with deionised H2O. All steps were performed at RT. The membrane was blocked with 5% blotto for at least 30-60 min then incubated for 1 h in 2.5% blotto with a 1:1000 dilution of probe (typically 1 μg/mL final concentration of protein or primary antibody) with constant shaking (45 rpm). The membrane was rinsed twice with TBS-T to remove excess blotto, followed by 3x 5 min incubations in TBS-T with constant shaking. If additional layers were required for HRP detection, the 1 h incubation was repeated with secondary or tertiary antibodies, typically at a 1:10,000 dilution in 2.5% blotto, followed by rinsing and washing as above. Immobilised protein-antibody complexes were visualised by chemiluminescence using ECL Western Blotting Detection Reagents (Amersham) and exposure to BioMax film (Kodak).

For re-probing with a different antibody, membranes were blocked in 5% blotto for 5 min, washed with deionised H2O and stripped by incubation in 0.2 M NaOH for 5 min with constant shaking (45 rpm). The membranes were washed with deionised H2O then blocked and probed as above.
xiii) Coupling of proteins to cyanogen bromide (CnBr)-activated sepharose

CnBr-activated sepharose (Amersham) was swelled and washed with 1 mM HCl through a sintered glass filter. The beads were transferred to a tube, allowed to settle and excess HCl removed. Protein at 2 mg/mL in 0.1 M NaHCO₃ pH 8.0, 0.5 M NaCl was added in a 3:1 ratio with washed CnBr beads and incubated for 1 h at RT with constant inversion by mixing. The beads were allowed to settle and the protein solution was removed and checked either by SDS-PAGE or UV spectroscopy to determine the coupling efficacy. Remaining active groups on the beads were blocked by incubation in 100 mM Tris-HCl pH 8.0 for 2 h. The beads were then washed 3x with PBS and stored in a 1:1 slurry at 4 °C in PBS containing 0.025% NaN₃.

xiv) Fluorescein Isothiocyanate (FITC) labeling of protein

As FITC is prone to photo bleaching, all steps were performed in low light or dark conditions. FITC was dissolved at 4 mg/mL in dimethyl sulfoxide (DMSO) and immediately used for coupling. The FITC solution was slowly added to a final ratio of 1:20 to protein at 2 mg/mL in PBS with gentle mixing. The reaction was incubated overnight at 4 °C in the dark. Unconjugated FITC was removed by extensive dialysis into PBS or by gel filtration through a G25 sephadex column using gravity feed. The efficacy of coupling was determined by UV spectroscopy of protein at 280 and 495 nm, where an absorbance ratio for 495:280 nm of between 0.3 and 0.7 was optimal.

xv) Cy5 labeling of protein

Cy5 labeling was performed using a FluoroLink™ Cy5 Reactive Dye kit (Amersham). Protein at 1 mg/mL was conjugated to Cy5 in 1 mL PBS overnight at 4 °C in the dark with constant mixing by inversion. Labeled protein was separated from unconjugated dye by passing through a 15 mL G25 sepharose column in low light conditions. The dye to protein ratio was typically ~0.8 Cy5 per molecule.

xvi) Seroconversion

Seroconversion was detected by Enzyme-Linked ImmunoSorbent Assay (ELISA). Protein (50 μL at 1 μg/mL in PBS) was allowed to bind to ELISA plate wells for 1 h at RT, followed by 3x washing with PBS-T. The wells were blocked by incubation with 100 μL of PBS-BSA for 1 h at RT and washing was repeated as above. Dilutions of human serum in PBS (50 μL) were added to wells in duplicate and incubated for 1 h at RT. Washing was repeated as
above. Wells were then incubated for 1 h at RT with 50 μL Goat anti human Ig-HRP diluted 1:1,000 in PBS-BSA, before further washing as above, with 3 additional washes in PBS. Protein:antibody complexes were detected by colour change after incubation of the plate with 50 μL per well of developing solution (50 mM citric acid, 100 mM Na₂HPO₄, 0.012% H₂O₂, 0.5 mg/mL o-phenylenediamine dihydrochloride) in the dark. The reaction was stopped by the addition of 50 μL 10% HCl per well and the colour change quantified by absorbance at 490 nm using a μQuant (BioTek instruments Inc.).

xvii) Production and purification of anti-SSL11 rabbit antibodies
A rabbit was maintained at the Animal Research Unit (now the Vernon Jansen Unit), Auckland, and injected with 100 μg of SSL11-US6610 allele in Freund’s Incomplete Adjuvant once a month for three months. The rabbit was culled after test bleeds showed high serum reactivity to SSL11 and the blood was collected by heart puncture. The blood was allowed to clot and the serum was recovered, filter sterilised, aliquoted, and stored at -20 °C for further use.

Immunoglobulins (Igs) were purified by passing reactive rabbit sera through a poros 20A (Protein A) column by FPLC. Bound fraction containing Igs was eluted with 10 mM glycine-HCl pH 2.0 into an equal volume of 100 mM Tris-HCl pH 8.0 and dialysed into PBS. The Igs were passed over a column of SSL11 immobilised to CnBr-sepharose to purify SSL11-specific polyclonal antibodies, eluted with 10 mM glycine-HCl pH 2.0 into an equal volume of 100 mM Tris-HCl pH 8.0 and dialysed into PBS for future use.

xviii) Pulldown assay
Buffy coat was obtained from the NZ Blood services and cells were prepared by ammonium chloride lysis followed by histopaque separation (see sections 2.2.4 i and ii). Poly or mononuclear cells were resuspended in 5 mL cell lysis buffer and briefly sonicated. The cell lysate was precleared through sepharose to remove sepharose-binding proteins, then loaded onto a 100 μL SSL11-US6610:sepharose column. The column was washed with 50 cv of ice-cold wash buffer, followed by 10 cv of TSA, 10 cv 50 mM Tris-HCl pH 6.8 and 10 cv water. Bound proteins were eluted in 2 cv of 10 mM sialic acid, which was then diluted 1:1 with 2x loading buffer and boiled at 94 °C for 2 min. Proteins were separated by SDS-PAGE (section 2.2.2 viii) and visualized by coomassie staining (section 2.2.2 xi).
Production and purification of recombinant, soluble FcαRI (rsFcαRI)

A mammalian cell line (266CHO K1 D8.10) which expresses and secretes FcαRI was kindly supplied by Dr. Bruce Wines (The Burnett Institute, Melbourne, Victoria, Australia). Cells were maintained in Complete RPMI 5% FCS supplemented with 50 mg/mL G418. For the purification of rsFcαRI, cells were expanded to ten T175 flasks (Becton Dickinson). The media was removed from adherent cells and replaced with 100 mL protein free media (JRH Ex Cell™ 325 PF CHO) per flask. The cells were cultured over 10 days. Cellular debris was removed by centrifugation and the supernatant filtered through a 0.22 μm filter. The supernatant was diluted 1:1 with PBS and passed through a 10 mL TALON™ (BD Biosciences) column equilibrated with PBS using gravity feed at 4°C. The column was washed with at least 20 cv of PBS followed by 10 cv of PBS with 5 mM imidazole. RsFcαRI was eluted with 5 cv PBS with 100 mM imidazole. Fractions were analyzed for purity by SDS-PAGE, pooled and stored at 4 °C.

Neuraminidase treatment of rsFcαRI

Terminal sialic acid was removed from recombinant FcαRI by treatment with neuraminidase (New England Biolabs). Approximately 10 μg of purified rsFcαRI was treated with 25 units of neuraminidase in a final reaction volume of 10 μL 1x G1 buffer (supplied by manufacturer). The reaction was incubated for at least 1 h at 37 °C with occasional mixing.

Enzymatic biotinylation of rsFcαRI using the engineered biotin target sequence

RsFcαRI was buffer exchanged by extensive dialysis into 10 mM Tris–HCl, pH 8.0, 7.5 mM MgCl₂, 5 mM NaCl. Biotin ligase, ATP (Amersham) and biotin (USB Corp.) were added to final concentrations of 5 μM (175 μg / mL), 5 mM and 1 mM respectively and incubated for 1 hr at 37 °C. Free biotin was removed by extensive dialysis of the protein into PBS at 4 °C in the dark. The efficacy of the reaction was tested by western blot using streptavidin-HRP.

Biosensor analysis of SSL11 interactions with rsFcαRI and sLeα

Biosensor analysis was performed on a BIAcore 2000. Ligands were coupled using carbodiimide chemistry to CM5 chips according to manufacturer’s instructions (BIAcore). Enzymatically-biotinylated rsFcαRI tagged at the C-terminus was captured (typically 200 RU) on a streptavidin coupled layer. BSA-sLeα (Dextra Laboratories, UK) was typically
coupled at 300 RU and blocked with BSA. Control channels for the subtraction of bulk and non-specific responses to give binding sensorgrams consisted of coupled streptavidin or BSA as appropriate. SSL11 (30 nM to 16 μM) dialysed into HBS-EP was passed over the immobilized ligands typically at 20 or 30 μL/min and the binding response plateau taken as a measure of the response at equilibrium (Req). HBS-EP was used as the running buffer in all reactions. SSL11-GL10 was regenerated with 4 M KCl and SSL11-US6610 was regenerated with 1 M MgCl₂. Equilibrium binding data was fitted to a Hill plot; \[ \frac{\text{Req}}{B_{\text{max}}} = \frac{[\text{SSL11}]^{nH}}{K_D + [\text{SSL11}]^{nH}}, \] where Req is the plateau binding response, \( K_D \) is the equilibrium dissociation constant, \( B_{\text{max}} \) is the maximal bound analyte at calculated saturation, \( n_H \) is the Hill coefficient and \( \frac{\text{Req}}{B_{\text{max}}} \) is the fraction of SSL11 bound.

For carbohydrate inhibition assays, 0.5 μM SSL11-GL10 was incubated with 10 mM of sugar molecules (carbohydrate kit obtained from Sigma) for 10 min at RT and injected over an rsFcαRI surface to detect changes in binding response compared to SSL11 alone. Reactions were performed in HBS-EP running buffer.

For IgA inhibition assays, human IgA (final concentration of 0.2 mg/mL) was preincubated with PBS or 0.2 mg/mL final concentration of TSST-1 or SSL7 for 10 min at RT prior to injection over an rsFcαRI surface. To investigate inhibition of binding due to SSL11, 0.2 mg/mL SSL11-GL10 was preinjected over the FcαRI surface for 3 min prior to injection of 0.2 mg/mL human IgA. Reactions were performed in HBS-EP running buffer.

xxiii) Preparation of glycan array samples
SSL11-US6610 was labeled with FITC (section 2.2.2 xiv) and prepared at a final concentration of 200 μg/mL in 20 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 0.05% v/v Tween 20 with 1% w/v BSA.

2.2.3 Protein Crystallography

i) Screening of crystallization conditions
Crystallization conditions were identified using a Cartesian HONEYBEE TM nanolitre dispensing robot (Genomic solutions TM) with an in-house 480-condition crystallization screen (Moreland et al., 2005). Crystals were grown in sitting drops in 96-well Intelliplates,
using 100 nL of protein solution mixed with 100 nL of precipitant solution. Manual fine screening was then performed around conditions that grew crystals. Bipyrimidal, native SSL11-US6610 crystals were grown by the sitting drop method at 18 ºC by mixing 1 μL protein (9.2 mg/mL in 20 mM Tris-HCl pH 7.4) with 1 μL 20% PEG 3350, 0.2 M NaH₂PO₄. Crystals appeared within 24 h and continued to grow for several days. SSL11-US6610:sLeˣ complex crystals were grown by the sitting drop method at 18°C by mixing 0.5 μL protein (15.8 mg/mL in 20 mM Tris-HCl pH 7.4) with 0.5 μL sLeˣ (10 mM in water – Dextra Laboratories, UK) and 1 μL 20% PEG 3350, 0.2 M tripotassium citrate. Crystals grew as diamond-shaped plates that appeared within 3 days and continued to grow for several weeks.

Attempts were made to prepare crystals of SSL11-US6610 in complex with sialic acid by a fine screen around the native crystallization conditions (0.2 M NaH₂PO₄, 18, 20 or 22% PEG 3350) with the addition of a final concentration of 0.2, 1, or 2 mM sialic acid. Drops were prepared using the sitting drop method at 18 ºC, by mixing 0.5 μL protein solution (15.8 mg/mL in 20 mM Tris-HCl pH 7.4), 0.5 μL 10 mM sialic acid, and 1 μL precipitant solution. Precipitate was visible immediately.

Attempts were made to prepare crystals of SSL11-US6610 in complex with FcαRI using 384 (robot screens 1-4) of the 480 in-house crystallization conditions (Moreland et al., 2005). Drops were prepared by the sitting drop method in 96-well Intelliplates, using 100 nL of protein solution (~6 mg/mL SSL11-US6610 and ~3 mg/mL FcαRI in 20 nM Tris-HCl pH 7.4) mixed with 100 nL of precipitant solution and storage at 18 ºC. Precipitate was immediately visible in 331 of the 384 conditions, with a further 35 conditions showing precipitation over the following 24 hours.

ii) Data collection and processing

Cryo conditions of precipitant solutions containing 10-30% glycerol, PEG 400 or ethylene glycol were screened for formation of ice rings and crystal stability at 113 K. Native SSL11 crystals were transferred to 70% paratone oil, 30% mineral oil and flash frozen in liquid nitrogen for data collection. X-ray diffraction data were collected at 100 K on beamline 9-1 at the Stanford Synchrotron Radiation Laboratory (Menlo Park, CA), using an ADSC Q 315 detector and a wavelength of 0.97907 Å. A complete data set was collected to 1.7 Å resolution and processed with HKL2000 software (Otwinowski and Minor, 1997).
The SSL11-US6610:sLe\textsuperscript{x} crystals were transferred to 70% paratone oil, 30% mineral oil and flash frozen in liquid nitrogen. X-ray diffraction data were collected at 113 K with an R-Axis IV image plate detector on a Rigaku MicroMax-007HF rotating anode generator at the University of Otago, New Zealand, using Cu-K\textsubscript{\alpha} radiation ($\lambda$ = 1.5418 Å). Data were collected to 1.44 Å resolution, but the highest resolution shells were incomplete, so the data set was truncated to 1.60 Å and processed with MOSFLM and SCALA from the CCP4 program suite (Leslie, 1992).

iii) Structure determination and refinement

The native SSL11-US6610 structure was solved by molecular replacement for a resolution range of 1.70-32.48 Å with Molrep (Vagin and Teplyakov, 2000), using the structure of SSL5 (Arcus et al., 2002) as a search model. The molecular replacement phases were then used with ARP/wARP (Perrakis et al., 2001), producing one continuous molecule (molecule C – residues 7 to 195) and three incomplete molecules (molecules A and B were missing loop regions 73 to 77 and 92 to 97 while molecule D was missing residues 66 to 82). Subsequent cycles of manual building in COOT (Emsley and Cowtan, 2004) and refinement in Refmac5 (Murshudov et al., 1997) using data in the resolution range 32.48 to 1.70 Å followed. Non-crystallographic symmetry (NCS) restraints between the four molecules were used in the initial rounds of refinement, replaced in later rounds by translation/libration/screw (TLS) restraints. Water molecules were included in the model if they had spherical density, with height greater than 3$\sigma$ in Fo – Fc difference maps and made favorable hydrogen bonding contacts with surrounding structure. One PO\textsubscript{4}\textsuperscript{3-} ion was modeled bound to each SSL11 molecule. The quality of the model was checked periodically with PROCHECK (Laskowski et al., 1993).

The SSL11-US6610:sLe\textsuperscript{x} complex structure was determined by molecular replacement using PHASER (McCoy et al., 2005), using the native SSL11-US6610 molecule C as a search model. This was followed by cycles of manual model building in COOT (Emsley and Cowtan, 2004) and refinement in Refmac5 (Murshudov et al., 1997). Clear, well-defined density was found for the complete sLe\textsuperscript{x} molecule but this was not added to the model until the protein structure was complete. A restraints library for sLe\textsuperscript{x} was developed through the program SKETCHER in the CCP4 suite. Water molecules were added as described above and one citrate ion was also modeled.
2.2.4 Cell biology

i) Erythrocyte lysis by ammonium chloride
In a 50 mL tube, 45 mL RBC lysis buffer was added to 5 mL fresh blood and mixed by inversion. The tube was incubated at 37 °C with occasional mixing by inversion until the solution became translucent. The cells were washed twice by centrifugation at 450 g for 5 min with low break and resuspension in PBS.

ii) Isolation of peripheral blood mono- and poly-nuclear cells
Fresh blood was collected in either sodium heparin or liquid K$_3$EDTA (for neutrophil rolling assays) vacutainer tubes (Becton Dickinson). Cells were separated by a buoyancy gradient as follows: 2.5 mL of Histopaque 1077 (Sigma Aldrich) was layered on top of 2.5 mL Histopaque 1191 (Sigma Aldrich) in a 15 mL Falcon tube (BD biosciences), with care not to disturb the interface. Freshly collected blood was carefully added on top (4-5 mL) and the tubes centrifuged at 450 g for 25 minutes with no brake. The separation resulted in layering of blood fractions with plasma, mononuclear cells, polynuclear cells and red blood cells / aggregate from top to bottom. Mononuclear cells include monocytes and lymphocytes, while the polynuclear cells were mainly neutrophils with some eosinophils. Generally, this preparation was relatively homogeneous, but in some instances, contaminating red blood cells were removed by mild salt lysis as follows. The cells were transferred to a 50 mL tube, resuspended in ~5 mL PBS and incubated with 20 mL of ice-cold 0.2% w/v NaCl for no more than 30 s before adding 20 mL ice-cold 1.6% w/v NaCl. The cells were recovered by centrifugation at 450 g for 5 min with low brake and the salt lysis step repeated if necessary. The cells were diluted with PBS and centrifuged at 450 g for 5 min low brake to recover the cells.

iii) Neuraminidase treatment of cells
Neutrophils (see section 2.2.4 ii) were washed once and resuspended at 1x10$^7$ cells/mL in 150 mM NaCl, 5 mM CaCl$_2$ pH 6.0. The cells were incubated with or without neuraminidase (25 unit/mL - New England Biolabs) for 1 h at 37° in a 5% CO$_2$ incubator with occasional mixing.
iv) Flow cytometry
To determine leukocyte populations that bound SSL11, cells were prepared by ammonium chloride erythrocyte lysis (see section 2.2.4 i). The cells were washed once and resuspended at $1 \times 10^7$ cells/mL in PBS-BSA. A 100 $\mu$L aliquot of cells was incubated for 15 min on ice with 0.1 $\mu$M FITC-labeled SSL11-US6610, washed twice and resuspended in 0.5 mL PBS-BSA. The cells were briefly vortexed and analysed by flow cytometry using a FACScan (Becton Dickinson) and gating of cell populations (i.e. granulocytes, monocytes and lymphocytes).

For competition assays by flow cytometry, neutrophils were isolated as described in section 2.2.4 ii, washed once and resuspended at $1 \times 10^7$ cells/mL in PBS-BSA, and aliquoted at 100 $\mu$L per sample. Each reaction was incubated with 5 $\mu$L of IgA-FITC (~5 $\mu$g), anti-human CD89 mAb (MIP8a-PE), or anti-sLe$^\alpha$ mAb (KM93), and 50 $\mu$L of SSL11-GL10 (100-3.125 $\mu$g and PBS-BSA for control) for 15 min on ice in the dark. The neutrophils were washed twice and resuspended in 0.5 mL PBS-BSA. Samples were briefly vortexed and staining was analysed on a FACScan (Becton Dickinson). Reactions were also performed on neuraminidase-treated cells.

To analyse neutrophil aggregation, cells were isolated as above and incubated for 10 min on ice with concentrations of SSL11-US6610 up to 4 $\mu$M. Cells were briefly vortexed and analysed by forward and side scatter through a FACScan (Becton Dickinson).

v) In vitro neutrophil rolling assay
Microchambers were prepared as follows. Capillary tubes (VD/3530-050 - Camlab, UK) were cleaned in 50% nitric acid by complete immersion (allowing the acid to fill the tube by capillary action) for 24 h in a fume cupboard. The tubes were blotted onto tissue paper to drain the nitric acid, rinsed with deionised H$_2$O and again blotted onto tissue paper. Anhydrous acetone was drawn through the tubes to remove traces of water, before filling the tubes by capillary action with 4% 3-Aminopropyltriethoxysilane (APES) w/v in anhydrous acetone and incubating for at least 30 s. The APES was removed from the tubes by blotting onto tissue and replaced with fresh 4% APES and incubating for at least 30 s. Anhydrous acetone was again drawn through the tubes before rinsing them with deionised water. The tubes were allowed to dry in a 5% CO$_2$ incubator. The microchambers were coated with
purified human P-selectin (Calbiochem) at 1 μg/mL in PBS for 1 h before blocking with 1% w/v BSA in PBS for 1 h just prior to use.

Neutrophils were purified from blood collected in K₃EDTA tubes (BD Biosciences) as described in section 2.2.4 ii. Cells were washed in Dulbecco’s PBS without Ca²⁺ (Sigma-Aldrich) before suspension at 1x10⁶ cells/mL in Dulbecco’s PBS containing Ca²⁺ and Mg²⁺ (Sigma-Aldrich). SSL11-US6610, anti-sLeα mAb (KM93) or PBS was incubated with 1 mL of neutrophils for 10 min on ice. The cell suspension was perfused through a microchamber at a rate of 0.3 mL/min (0.8 dyn/cm²) at RT by a Harvard Apparatus syringe pump. Cell binding to the microchambers was visualized with an Axiovert S100 microscope over 5 separate fields after 3 minutes of perfusion. Digital images were captured with an Axiocam MR-3 camera and analyzed with Axiovision 4.5 software.

vi) Preparation of slides for microscopy
For aggregation assays, neutrophils (see section 2.2.4 ii) were resuspended in PBS at 1x10⁶ cells/mL. To 100 μL aliquots, SSL11-US6610 was added at 0, 0.1, 0.25, 0.5 or 0.75 μM, gently mixed and incubated for 10 min on ice. Samples were diluted 1:1 with 0.4% trypan blue in PBS, vortexed briefly, loaded onto a hemocytometer and visualized by bright field microscopy.

For fluorescence microscopy, neutrophils (see section 2.2.4 ii) were added to an 8-chamber slide (Becton Dickinson) at 5x10⁵ cells/well in Dulbecco’s PBS with Ca²⁺ and Mg²⁺ and allowed to adhere for 30 min at 37° in a 5% CO₂ incubator. The cells were washed 3x with 150 mM NaCl, 5 mM CaCl₂ pH 6.0. Adherent neutrophils were incubated with or without neuraminidase (25 units/mL – New England Biolabs) in 150 mM NaCl, 5 mM CaCl₂ pH 6.0 for 1 h at 37° in a 5% CO₂ incubator. The cells were washed 3x with PBS then incubated with 100 μL 0.1 μM FITC-labeled SSL11-US6610 in PBS for 5 min on ice. The cells were washed 3x with PBS, fixed with 4% paraformaldehyde in PBS for 10 min at RT, washed 3x with PBS, air dried, mounted in Prolong® Gold with DAPI (Invitrogen) and visualized by fluorescent microscopy.

For confocal microscopy, freshly isolated human neutrophils (section 2.2.4 ii) were added to an 8-chamber slide (Becton Dickinson) at 5x10⁵ cells/well in Dulbecco’s PBS with Ca²⁺ and
Mg$^{2+}$ and allowed to adhere for 30 min at 37° in a 5% CO$_2$ incubator. The cells were washed 3x with ice-cold PBS-BSA then incubated with 100 μL per well of either 0.1 μM Cy5-labeled SSL11-US6610 in PBS-BSA or 0.1 μM Cy5-labeled SSL11-T168P in PBS-BSA for 5 min on ice or 30 min at RT. Washing with ice-cold PBS was repeated three times. The cells were fixed with 4% paraformaldehyde in PBS for 10 min at RT, washed 3x with PBS, air dried, mounted in Prolong® Gold with DAPI (Invitrogen) and visualized by confocal laser scanning microscopy on a Leica TCS SP2.

vii) Two-dimensional gel analysis

Neutrophils were purified as described in section 2.2.4 ii and resuspended at 1.25x10$^7$ cells/mL in RPMI 1640 supplemented with 10 mM HEPES-HCl pH 7.6 and 1% heat inactivated platelet poor plasma (PPP). PPP was prepared by centrifugation of whole blood collected in glass vacutainer tubes (Becton Dickinson) at 400 g for 5 min and heat inactivated by incubation at 56 °C for 30 min. Samples of 2 mL volume (2.5x10$^7$ cells) were incubated at 37 °C in a 5% CO$_2$ incubator for 1 h, with or without 0.1 μM SSL11-US6610. The neutrophils were then washed 3x with 0.34 M sucrose, 1 mM EDTA, 10 mM Tris-HCl pH 7.6 to remove salts and surface-bound proteins. After the final wash, cells were resuspended in 360 μL rehydration buffer (7 M urea, 2 M thiourea, 2% w/v CHAPS, 10 mM DTT, 2% w/v Ampholytes pH 3-10, 0.01% w/v bromophenol blue) and contained an estimated 500 μg total protein per sample.

Two-dimensional gel electrophoresis was performed by the Centre for Genomics and Proteomics (the University of Auckland, New Zealand). Protein samples were used to rehydrate isoelectric focusing (IEF) gels overnight (18 cm, pH 3-10 nonlinear Immobiline DryStrip IEF gels; Amersham Pharmacia Biotech). IEF was performed at 20 °C to 60 kVh under mineral oil, followed by 2x 15 min SDS equilibration steps (DTT followed by iodoacetamide (IAM)-containing equilibration buffers; 5 M Urea, 30% v/v glycerol, 0.05 M Tris-HCl pH 6.8, 30 mM SDS, containing first 8 mg/mL DTT, and second 36 mg/mL IAM). Proteins were separated by size on 10-14% gradient polyacrylamide slab gels by vertical electrophoresis (Amersham Pharmacia Biotech) at 1000 V. Protein spots were visualized by agitation in colloidal Coomassie Brilliant Blue G-250 for 16 h, followed by destaining in deionized water for 20 h.
Chapter 3 – Investigating the interaction between SSL11 and FcαR1

3.1 Introduction

The translated amino acid sequences of SSL11 from published genomes (Baba et al., 2002; Copeland et al., 2006a, b; Diep et al., 2006; Gillaspy et al., 2006; Herron-Olson et al., 2006; Kuroda et al., 2001; Roe et al.) have a high variation between alleles i.e. between 59% and 93% identity. However, the ssl11 gene has been found in all sequenced strains, indicating a selective preservation mechanism. This poses the questions – are the SSL11 alleles still the same protein and do they have the same function? Previous work by Dr. Ries Langley found that one allele, designated GL10, was able to bind the myeloid receptor FcαRI (CD89) and inhibit binding of its native ligand, IgA. This section addresses work done at the DNA and protein level to characterise SSL11 and its interaction with the known ligand, FcαRI.

3.2 Analysis of ssl11 from clinical isolates of S. aureus

To investigate the degree of allelic variation in the ssl11 gene, it was amplified by PCR and sequenced from several unrelated clinical isolates of S. aureus. The ssl11 gene was first isolated from the GL10 strain and cloned into the pET32a3C expression system by R. Langley (2003). The plasmid DNA from this construct provided a positive control for the amplification of ssl11 from other clinical isolates of S. aureus. Five SSL11-specific primers were designed from published sequences (Baba et al., 2002; Kuroda et al., 2001) (see appendix 7.1). The primers were designed to amplify ssl11 without the predicted signal peptide as determined by SignalP (Bendtsen et al., 2004). An example of an agarose gel electrophoresis of ssl11 amplified from 28 clinical isolates by PCR is presented in figure 3.1.
Agarose gel electrophoresis of PCR products amplified from various clinical isolates of *S. aureus* using the primers SSL11-N315-Fw and SSL11-N315-Rv. The product runs at approximately 650 bp as indicated by the arrows.

The PCR products from 44 clinical isolates were sequenced. Two distinct clades were defined by alignment of the translated amino acid sequence using Clustal W (Thompson *et al.*, 1994) and graphical representation by a Neighbour-Joining (NJ) tree (figure 3.2). This distribution is due to a recombination event in some *S. aureus* strains between the 5' end of *sslII* and the upstream *HsdM* gene (Fitzgerald *et al.*, 2003). Alleles were between 194 and 202 amino acids in length and 22.4 kDa to 23.3 kDa in size with calculated isoelectric points (pI) of between 5.5 and 8.8. The alleles share between 56.3% and 93% amino acid sequence identity (figure 3.3). Out of the 44 isolates sequenced, 40 (91%) have undergone the recombination event with *HsdM* (branches 1-5), while only 4 (9%) of the alleles have not (branches 6-8).

There was no obvious correlation between the SSL11 alleles and either the geographical distribution or susceptibility of the producing strain to antibiotics. For example, the JH1 strain is vancomycin susceptible, while the JH9 strain is vancomycin resistant (Sieradzki *et al.*, 2003), but both SSL11 alleles are in the same branch. Similarly, the SSL11 allele from the meticillin resistant strain MW2 and the meticillin sensitive strain, MSSA476 are in branch 6 (figure 3.2)
The amino acid sequences of 44 SSL11 alleles were aligned with 12 amino acid sequences available from genome projects (underlined) using Clustal W. From this data, an N-J tree was constructed, showing the clustering of eight allele types (numbered on the branches) within two distinct clades. The origin of the clinical isolates giving rise to the SSL11 sequences are represented by colour as follows: USA, Japan, United Kingdom, Germany, New Zealand. Bold indicates a strain isolated from a bovine source.
Figure 3.3 - Variation between SSL11 alleles

A) Amino acid sequence alignment by Clustal W of SSL11 alleles lacking the signal peptide determined by SignalP (Bendtsen et al., 2004), using the numbering system as per figure 3.2. B) Table showing the amino acid sequence identity between SSL11 alleles as a percent, using the numbering system as per figure 3.2.
3.3 Cloning, expression and purification of SSL11

To investigate the functional similarities or differences between SSL11 alleles, ssl11 genes were cloned and expressed. The ssl11 gene was amplified from *S. aureus* strain US6610 (allele type 2) genomic DNA and protein was expressed as a thioredoxin-3C fusion protein using the pET32a3C system (figure 3.4). This allele is 66.3% identical to the GL10 allele in amino acid sequence (Langley, 2003). SSL11-US6610 was separated from thioredoxin (TrxA) by the 3C protease from human rhinovirus that cleaves the sequence EVLFQ/GP (Walker *et al.*, 1994). Because a *BamHI* site is introduced 3′ of the 3C protease recognition sequence, the result is in an additional four amino acids (*i.e.* GSGP) at the N-terminus of all proteins expressed using the pET32a3C and pGEX3C plasmids.

![Figure 3.4 - The purification of SSL11-US6610](image_url)

**Figure 3.4 - The purification of SSL11-US6610**

Separation of proteins on 12.5% SDS polyacrylamide gels showing the purification stages of SSL11-US6610. SSL11-US6610 expression was induced by IPTG in AD494(DE3)pLysS *E.coli*. (A) Bacterial lysate was loaded onto an IDA sepharose column, washed and eluted in three fractions. (B) Elution fractions containing recombinant protein were pooled, digested with 3C protease, dialysed to remove imidazole and passed back through an IDA sepharose column. Purified SSL11 was eluted in the flowthrough.

Yields of up to 30 mg of soluble fusion protein were typical from 1 L of liquid culture. Yields of 5-10 mg purified SSL11-US6610 per L of liquid culture were obtained after cleavage by 3C protease and separation of proteins by size exclusion. Both SSL11-GL10 and SSL11-US6610 were stable in phosphate, Tris and HEPES buffers. However, the polydispersity of SSL11-GL10 improved in buffers containing salt as determined by dynamic light scattering. SSL11-US6610 was typically produced in batches of 20-100 mg for crystallography trials.
(see chapter 4) and stored for extended periods in Tris-HCl pH 7.4 at -80 °C, or 4 °C for immediate use. SSL11-GL10 was typically produced for immediate use and stored in Tris-HCl pH 7.4, 150 mM NaCl at 4 °C.

Expression of recombinant SSL11 from *S. aureus* strains ATCC 33593 (allele type 4) and NU4483 (allele type 1) was also attempted. Expression of the full length TrxA-3C-SSL11 fusion protein was evident in IPTG-induced bacteria which were lysed and immediately run on SDS-PAGE (figure 3.5A). Purification by IMAC routinely resulted in a prominent 3C protease-sensitive ~20 kDa protein, but not the expected full length fusion protein. Treatment of the ~20 kDa protein reduced its size by 2-3 kDa (figure 3.5B), suggesting that these alleles

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**Figure 3.5 - SDS-PAGE of two alleles of SSL11 expressed using the pET32a3C system**

A) *E. coli* expressing *ssl11* amplified from two strains (NU4483 and ATCC 33593) were harvested before (U) and after induction (I) with IPTG, boiled and separated by 12.5% SDS-PAGE. B) Fusion protein was purified from bacterial lysate by an IDA column (-), digested with 3C protease (+) and proteins were separated by 12.5% SDS-PAGE. M denotes the molecular weight ladder.
contained a highly-sensitive proteolytic site located somewhere in the N-terminus. Therefore, further investigations focused on the successfully expressed alleles, SSL11-GL10 and SSL11-US6610.

3.4 Comparison between SSL11 from GL10 and US6610

Mature, secreted SSL11-GL10 is 197 amino acids in length, has a theoretical pI of 5.50 and a molecular mass of 22543 Da, whereas SSL11-US6610 is 196 amino acids in length, has a theoretical pI of 7.85 and a molecular mass of 22651.2 Da. Dynamic light scattering indicated that SSL11-GL10 formed a dimer at concentrations as low as 2 mg/mL, whereas SSL11-US6610 remained monomeric at concentrations greater than 9 mg/mL.

3.5 Seroconversion to SSL11

To determine whether SSL11 is expressed by *S. aureus* in human infection, human sera obtained from eight healthy donors and four patients admitted to hospital suffering from acute *S. aureus* infection (see appendix 7.4) were tested for the presence of specific antibodies against SSL11-GL10 and SSL11-US6610, using SSL5 as a control against cross-reactivity (table 3.1). Donor RL was unique, having previously worked on SSL5 in the laboratory, explaining his higher antibody titre to SSL5 and donor MC is myself, explaining the higher titre to both alleles of SSL11.

Titres were calculated by a dilution series of sample serum, where the titre was taken as the dilution required for Abs(490)=0.1. Titres greater than 1:6400 were converted to a standard 1:12800 for statistical analyses, although in most cases, the expected titres would be much higher (see appendix 7.5). The observed titres between SSLs were significantly different *i.e.* SSL5 gave a P value of 0.007 to both SSL11 alleles and SSL11-GL10 gave a P-value of 0.0002 to SSL11-US6610 (figure 3.6A). This indicates that the specific antibodies are not cross-reactive. The four patients’ sera showed significantly lower overall titres of reactive antibodies (P=0.009) compared to the eight healthy donors (figure 3.6B). These values are likely to be more significant, as titres greater than 1:6,400 are expected to be much higher than the standardized value of 1:12,800.
Table 3.1 - Detection of reactive sera against SSL5 and SSL11 by ELISA
An arbitrary cutoff of Abs(490)=0.1 was used to compare the titres of specific antibodies against SSL5 and SSL11 from 12 donors.

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Figure 3.6 - Statistical analysis of seroconversion results
A) Bar chart of the mean antibody titres against the individual SSLs, with error bars indicating ± standard error of the mean (SEM). B) Bar chart of the combined mean antibody titres between healthy and patient donors. Error bars indicate ± SEM. ** indicates P<0.01 and *** indicates P<0.001.

3.6 Binding studies of SSL11
SSL11-GL10 inhibited IgA binding to leukocytes by directly binding to the myeloid IgA receptor, FcαRI (Langley, 2003). A stably transfected CHO cell line that secretes a soluble, recombinant form of FcαRI was obtained from Dr. Bruce Wines (unpublished) to further
investigate this interaction. This construct was modified to contain a His6 and biotinylation target sequence at the C-terminus of FcαRI (see appendix 7.2).

3.7 Cloning, expression and purification of biotin ligase (BirA)
For the purpose of biotinylating FcαRI and other constructs containing a biotinylation target sequence, the enzyme BirA was cloned from and expressed in *E. coli*, using the expression vector pGEX3C (figure 3.7A). Fusion protein was cleaved by 3C protease and BirA was subsequently purified by ion exchange chromatography (figure 3.7B). The functional activity of BirA was tested using purified rsFcαRI containing the biotin target sequence LHHILDAQKMVWNHR, where biotin is covalently added to the lysine. Figure 3.6C indicates successful biotinylation of rsFcαRI and detection by streptavidin-HRP.

![Image](image1.png)

**Figure 3.7 - Expression and purification of BirA**
A 12.5% SDS-PAGE of (A) the expression of BirA using pGEX3C and B) the eluted product pooled, cleaved with 3C protease and separated by anion exchange (proteins marked with arrows). C) Western analysis of FcαRI biotinylated using BirA and probed with streptavidin-HRP.

3.8 Biosensor analysis of human IgA binding to an FcαRI surface
Previous results from our lab showed that both SSL7 and SSL11-GL10 compete with IgA binding to leukocytes in a dose-dependent manner (Langley, 2003). Therefore, biosensor analysis was performed to examine the SSL11-FcαRI interaction. RsFcαRI was biotinylated using BirA and reacted with streptavidin coupled to a CM5 chip (BIAcore) by carbodiimide chemistry. This ensures consistent orientation of rsFcαRI molecules on the chip surface.
Complete inhibition of IgA binding to immobilised FcαRI was observed when IgA was preincubated with SSL11 (figure 3.8). Because SSL11-GL10 binds directly to FcαRI but not IgA (Langley, 2003), SSL11-GL10 was reacted with the FcαRI surface immediately prior to an injection of IgA. This resulted in a ~50% inhibition of IgA binding. The SAg, TSST-1 as a control protein, had no effect on the binding of IgA to immobilised FcαRI.

Figure 3.8 - Biosensor analysis of IgA binding to an FcαRI surface
Sensorograms showing the response from binding of human IgA to FcαRI after pretreatment with PBS, SSL7, TSST-1 or pre-treatment of the FcαRI surface with SSL11-GL10.

3.9 Steady state affinity of SSL11 binding FcαRI
The dissociation constants (K_D) of SSL11-GL10 and SSL11-US6610 binding to FcαRI were determined by steady state affinity using biosensor analysis (figure 3.9). A series of SSL11 concentrations were injected over an FcαRI surface and the response at equilibrium (Req) plotted against the concentration as a fraction of the maximum response (Bmax) using the Hill equation, Req/Bmax = ([rSSL11]^nH)/(K_D + [rSSL11]^nH), where nH is the Hill coefficient.
Figure 3.9 - Steady state curves of SSL11 binding FcαRI

Various concentrations of A) SSL11-GL10 and B) SSL11-US6610 were injected in triplicate over an FcαRI surface (16 μM, 8 μM, 4 μM, 2 μM, 1 μM, 0.5 μM, 0.25 μM, 0.125 μM). The values at Req for both alleles of SSL11 are shown on a single plot standardised by fraction bound, where B_max is y=1 and the lines indicate model fitting using the Hill equation.

The K_D is determined as the concentration of ligand at which the fraction bound is 50% of the total ligand. SSL11-GL10 was calculated to have a K_D of 9.02 ± 1.00x10^-6 M with a Hill coefficient of 0.93 ± 0.03. SSL11-US6610 had a K_D of 0.72 ± 0.05x10^-6 M with a Hill coefficient of 1.01 ± 0.08 (all values ± SEM). The affinity of bound ligand molecules (SSL11) to its receptor (FcαRI) is by definition, not affected by other bound ligand molecules if the Hill coefficient is 1. Hill coefficients smaller than 1 indicate negative cooperativity, where affinity for the receptor decreases as more ligand is bound. Conversely, Hill coefficients greater than 1 indicate positive cooperativity, where affinity for the receptor
increases as more ligand molecules bind. The micromolar $K_D$ between SSL11 and FcαRI are consistent with physiological interactions between cell adhesion molecules (Ji et al., 2003).

### 3.10 Carbohydrate inhibition of SSL11 binding to FcαRI

Previous studies found that SSL11 only bound to FcαRI when expressed by mammalian cells and not insect cells (Sf9) infected with recombinant baculovirus (Langley and Wines, unpublished). Because the primary difference between protein expressed in these two systems is their post-translational glycosylation (Altmann et al., 1999), the effect of sugars on the interaction between SSL11 and FcαRI was investigated.

SSL11 preincubated for 10 min with 10 mM of individual carbohydrates at RT showed no significant difference in binding to an FcαRI surface compared to SSL11 alone (figure 3.10A). However, 10 mM sialic acid (N-acetylneuraminic acid) completely regenerated the flow cell surface after an injection of SSL11 (Figure 3.10B). Proteins expressed in insect cells lack N-linked sialation, compared to mammalian cells (Altmann et al., 1999), which supports the previous observation by Langley and Wines (unpublished). The inhibitory effect was only observed when sialic acid was in water and not buffered. The inhibition of SSL11 binding to FcαRI by sialic acid indicates that it may be involved in the binding interaction.
Figure 3.10 - The effect of carbohydrate on the interaction between SSL11 and FcαRI

A) Sensorgrams of the response of interaction between FcαRI and SSL11-GL10 when preincubated with or without 10 mM carbohydrate for 10 min at RT. B) An injection of 10 mM sialic acid in water is able to remove all bound SSL11-US6610 from the FcαRI surface.
Sialic acids are nine-carbon sugars that are important molecules involved in cell-cell interactions, regulating receptor signaling and other immune processes. The two most common forms of sialic acid are N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) (figure 3.11). Humans have lost the ability to express Neu5Gc (Varki, 2001), therefore, sialic acid (Sia) will be used to refer to Neu5Ac for the remainder of this thesis.

Figure 3.11- Sialic acids
The structures of the two most common forms of sialic acid A) Neu5Ac and B) Neu5Gc
3.11 SSL11 binding to FcαRI is Sia dependent

From the biosensor results, Sia was implicated in the binding between SSL11 and FcαRI. To investigate this further, purified rsFcαRI was treated with neuraminidase and probed with SSL11-US6610 and anti-SSL11-US6610 rabbit antibodies by Western blot (figure 3.12 top panel). The disappearance of bands in the neuraminidase-treated FcαRI samples suggested that terminal sialation was necessary for SSL11 binding. To confirm that the disappearance of FcαRI bands in the neuraminidase-treated samples was not due to proteolytic activity and that equal amounts of protein were loaded, the membrane was stripped and re-probed with the anti-FcαRI mAb, MIP8a (Figure 3.12 bottom panel). The slight shift in neuraminidase-treated samples results from the loss in mass associated with Sia.

![Western analysis of SSL11 binding to FcαRI](image)

**Figure 3.12 - Western analysis of SSL11 binding to FcαRI**

Samples of FcαRI treated (+) or untreated (-) with neuraminidase were probed using SSL11-US6610 (top panel) and MIP8a (bottom panel) by western blot.
3.12 SSL11-US6610 binds to leukocyte populations

SSL11-GL10 was shown to bind strongly to granulocytes, monocytes and weakly to a population of lymphocytes (Langley, 2003). The leukocyte binding profile of SSL11-US6610 labeled with FITC was investigated by flow cytometry. Leukocytes were prepared by erythrocyte lysis and gated for the various cell populations (figure 3.13A). Granulocytes (figure 3.13B) and monocytes (figure 3.13C) stained with similar intensity, while lymphocytes separated into two populations of moderate and weakly binding cells (figure 3.13D). The staining profile is consistent with SSL11-GL10. FcαRI is present on both monocytes and granulocytes, but is absent on lymphocytes, indicating that SSL11 binding was not specific for FcαRI.

Figure 3.13 - Flow cytometry of leukocytes

A) Leukocyte populations gated by forward and side scatter ■ - granulocytes, ▶ - monocytes, ▵ - lymphocytes. (B) Granulocytes, (C) monocytes, and (D) lymphocytes stained with 0.1 μM SSL11-US6610-FITC, where ▶ indicates cells similarly gated but not stained with SSL11-US6610-FITC.
3.13 SSL11-GL10 inhibits IgA binding to neutrophils

Biosensor results indicated that SSL11 inhibited IgA binding to FcαRI (section 3.8). This effect was further investigated using flow cytometry of neutrophils. SSL11-GL10 effectively blocked the binding of IgA-FITC to purified human neutrophils in a dose-dependent manner, with a maximal concentration of ~30 μM tested (100 μg SSL11-GL10/sample - Figure 3.14A). Likewise, SSL11-GL10 also inhibited the binding of the anti-FcαRI mAb, MIP8a (figure 3.14B). MIP8a is an antibody that binds to the IgA binding site on FcαRI. Neutrophils pretreated with neuraminidase were no longer susceptible to MIP8a blocking by SSL11-GL10 (figure 3.14C), confirming that SSL11 binding was dependent on Sia.

Figure 3.14 - Inhibition of IgA and anti-FcαRI mAb binding to neutrophils by SSL11-GL10
A) Histogram showing the effects on staining of neutrophils with IgA-FITC by increasing amounts of SSL11-GL10. Histograms showing the effects on staining with MIP8a in (B) mock treated and (C) neuraminidase-treated neutrophils by increasing amounts of SSL11-GL10. SSL11-GL10 was added to samples as indicated: 0 = 0 μg, a = 3.125 μg, b = 6.25 μg, c = 12.5 μg, d = 25 μg, e = 50 μg, f = 100 μg.
3.14 SSL11-US6610 but not SSL11-GL10 causes neutrophil aggregation

The competition experiments presented in section 3.13 could not be repeated using SSL11-US6610 because of cellular aggregation. This effect was directly observed by flow cytometry of neutrophils incubated with increasing concentrations of SSL11-US6610 (figure 3.15A-F) to 4 µM. The population of normal-sized neutrophils decreased with increasing concentrations of SSL11-US6610 and a population with smaller forward and side scatter increased proportionally. The counts per second decreased with increasing concentration of SSL11-US6610 (from ~1000/s to ~50/s). This indicated that the neutrophils were aggregating and unable to pass through the flow cytometer aperture. The appearance of a new population with lower forward and side scatter is most likely due to the depletion of neutrophils by aggregation and the increase in the proportion of non-neutrophils.

To confirm that the neutrophils were aggregating, purified neutrophils were incubated with various concentrations of SSL11-US6610 on ice for 10 min, vortexed briefly and examined by bright field microscopy in the presence of trypan blue (figure 3.15G-J). There was clear evidence of cellular clumping at 0.5 µM of SSL11-US6610, while no visible effect was noted at 0.25 µM or 0.1 µM. Thus one significant difference was observed between SSL11-GL10 and SSL11-US6610, as SSL11-GL10 does not aggregate neutrophils at the highest concentration used in the inhibition studies (~30 µM – section 3.13).
Figure 3.15 - Neutrophil aggregation by SSL11-US6610

Neutrophils were examined by flow cytometry for forward and side scatter after incubation with SSL11-US6610 at a final concentration of (A) 0 μM, (B) 0.25 μM, (C) 0.75 μM, (D) 1 μM, (E) 2 μM, or (F) 4 μM and presented above as dot blots. Purified neutrophils were incubated with (G) 0.1 μM, (H) 0.25 μM, (I) 0.5 μM, or (J) 0.75 μM SSL11-US6610 and visualised by bright field microscopy.
3.15 Discussion

A high degree of allelic variation was found in the \textit{ssl11} gene. Two distinct clades exist (figure 3.2) due to a recombination event between the 5' end of \textit{ssl11} and the upstream \textit{HsdM} gene, resulting in highly variable N-terminal sequences (figure 3.16). The two clades of SSL11 are divided according to the numbering system in figure 3.2 as follows - alleles 6-8 are the more primitive forms, while alleles 1-5 have undergone the recombination event. The result is low overall identity between alleles, ranging from 56-93%, where identity within clade 1 (alleles 1-5) is 59-76% and clade 2 (alleles 6-8) is 80-93%. It is interesting that a higher degree of variation is observed in the alleles which have undergone recombination with \textit{HsdM}. It is possible that these strains are evolving at a more rapid rate to compensate for selective environmental and host pressures.

![Figure 3.16 - Recombination in ssl11](image)

A Haplot analysis of the genetic region including and surrounding \textit{ssl11} from six strains of \textit{S. aureus}, representing SSL11 alleles 1, 4, 6 and 8. Polymorphic nucleotides are drawn as vertical black lines and putative areas of recombination are drawn in grey (modified from Fitzgerald \textit{et al.}, 2003).

Two alleles of SSL11 studied here, from \textit{S. aureus} strains GL10 and US6610, share 66.3% amino acid sequence identity. Both alleles expressed in high yields and were used for further investigation. Seroconversion studies with SSL11-GL10, SSL11-US6610 and SSL5 indicate that these proteins are immunogenic (table 3.1), confirming that these proteins are produced by \textit{S. aureus}. Additionally, all individuals tested had developed antibodies to SSL11, indicating it is a common antigen of \textit{S. aureus}. Titres for SSL11-GL10, SSL11-US6610 and SSL5 were significantly different, indicating minimal cross reactivity. The comparatively higher titres of antibodies against SSL11-GL10 compared to SSL11-US6610 may be a result of the increased frequency of \textit{S. aureus} strains that produce SSL11-GL10 (\textit{i.e.} 6/56 and 1/56 of strains identified from clinical isolates respectively). The four patient samples exhibited significantly lower antibody titres when compared to healthy donors (P=0.009). Although this...
represents a very small study of seroconversion rates, this significant difference suggests a larger study is required to assess if low, non-neutralising antibody titres correlates to an increased susceptibility to *S. aureus* infection.

Both SSL11-US6610 and SSL11-GL10 stained intensely for granulocytes and monocytes, with moderate staining of a lymphocyte sub-population. Both alleles exhibited direct binding to the myeloid receptor FcαRI, which is involved in important immune processes including phagocytosis, degranulation, respiratory burst and cytokine release upon activation by IgA (Monteiro and Van De Winkel, 2003). Notably, the interaction between SSL11 and FcαRI was found to be completely dependent on sialation of FcαRI. The *K*<sub>D</sub> of SSL11-GL10 binding to FcαRI was 9.02 ± 1.00x10<sup>-6</sup> M compared to 0.72 ± 0.05x10<sup>-6</sup> M for SSL11-US6610, a 12.5–fold difference. Both alleles had Hill coefficients of ~1, indicating that binding of SSL11 molecules was not affected by bound SSL11. Consistent with the higher affinity to FcαRI, SSL11-US6610 readily aggregated neutrophils at concentrations above 0.5 μM, whereas SSL11-GL10 showed no observable aggregation up to concentrations of 30 μM. Given the above results, the indication is that although the sequence identity is only 66.3%, the two proteins appear to be allelic variants but they also exhibit some functional variation. Because SSL11-US6610 exhibited a higher affinity to FcαRI than SSL11-GL10, future work primarily focused on the US6610 allele where possible.
Chapter 4 – The three-dimensional structure of SSL11 and its interaction with sLe\textsuperscript{x}

4.1 Introduction
The bacterial SAg fold comprises an N-terminal OB domain and C-terminal β-grasp domain (see section 1.5.3 i). This fold provides a stable scaffold that supports a high variation in amino acid sequence; identity for SSLs and SAgs is typically between 20 and 30% on a pairwise basis. The crystal structures of two SSL proteins have been solved to date - SSL5 (Arcus et al., 2002) and SSL7 (Al-Shangiti et al., 2004), which share structural homology but functional distinction with the bacterial SAgs.

The interaction between SSL11 and the tetrasaccharide sialyl Lewis X (sLe\textsuperscript{x}: Neu5Ac\(\alpha\)2-3Gal\(\beta\)1-4[Fuc\(\alpha\)1-3]GlcNAc), was first suggested by a collaborator, Dr Bruce Wines (unpublished). Because sLe\textsuperscript{x} is a key component of the leukocyte recruitment pathway (see section 1.3.3 i), the binding of SSL11 to sLe\textsuperscript{x} was further investigated. This chapter presents structural data on SSL11-US6610 and the functional and structural characterisation of its interaction with sLe\textsuperscript{x}.

4.2 Crystallization of SSL11-US6610
The quality of purified SSL11-US6610 for crystallization trials was assessed by dynamic light scattering and gave a polydispersity index (Cp/Rh) of 20.8%. Crystals formed in nine conditions from a 480-condition screen (Moreland et al., 2005) (see section 2.2.3 i) and several of these conditions were fine-screened for crystal optimisation (figure 4.1A-B). However, these crystals were typically needles and often grew in clusters. A single, small, polyhedral crystal of 0.08x0.05 mm in the visible plane, formed in 0.2 M NaH\textsubscript{2}PO\textsubscript{4}, 20% PEG 3350 (figure 4.1C). Fine-screening around this condition ultimately revealed that higher quality crystals could be grown in this condition by using larger drops. Large polyhedral crystals (>0.2 mm in all dimensions) were grown in sitting drops of 1 \(\mu\)L protein (9.2 mg/mL) and 1 \(\mu\)L 0.2 M NaH\textsubscript{2}PO\textsubscript{4}, 20% PEG 3350 at 18 °C. Crystals were visible within 24 h and continued to grow for several days (figures 4.1D-E).
Figure 4.1 - SSL11-US6610 crystals

(A) SSL11-US6610 crystals grown in 0.2 M Li₂SO₄, 8% mmePEG 550, 8% PEG 20k have a “broccoli” appearance. (B) Addition of 0.1 M (NH₄)₂SO₄ produced better-defined crystals. (C) Crystals grown in 0.2 M NaH₂PO₄, 20% PEG 3350 formed polyhedral crystals in the initial 0.2 μL drop screen. (D) The 2 μL drop that produced X-ray diffraction-quality crystals. (E) Bipyramidal crystals also formed in this condition and are imaged under polarised light.

4.3 X-ray diffraction data from SSL11-US6610 crystals

Cryo conditions tested for flash-freezing of SSL11-US6610 included glycerol, ethylene glycol and low molecular weight PEG (section 2.2.3 ii). Typically, transfer of crystals into these conditions resulted in the crystal dissolving or shattering into smaller fragments. A data set was collected to 2.10 Å resolution at the Institute of Molecular Biosciences (Massey University, New Zealand) from a broken crystal prepared by diffusing small amounts of glycerol into the protein drop. However, these data were superseded by a superior data set collected from crystals flash-frozen in 70% paratone oil, 30% mineral oil, which diffracted to 1.70 Å resolution at the Stanford Synchrotron Radiation Laboratory (Menlo Park, CA).
4.4 Determination and refinement of the SSL11-US6610 crystal structure

The crystals were monoclinic with a space group P2₁. The unit cell dimensions were \( a = 54.35 \, \text{Å}, \quad b = 98.15 \, \text{Å}, \quad c = 79.51 \, \text{Å}, \quad \beta = 91.5^\circ \). The unit cell had a Matthews coefficient of 2.2 \( \text{Å}^3 \, \text{Da}^{-1} \) for four molecules per asymmetric unit with 44.6% solvent content.

The structure of SSL11-US6610 was solved by molecular replacement using Molrep and an initial model was built with Arp/wARP (section 2.2.3 iii). The initial model comprised one continuous molecule (molecule C – residues 7 to 195) and three incomplete molecules; molecules A and B were missing residues 73 to 77 and 92 to 97 while molecule D was missing residues 66 to 82. After cycles of manual rebuilding using COOT (Emsley and Cowtan, 2004) and refinement using Refmac5 (Murshudov et al., 1997), molecules A and D were complete from residue 6 to 196 and molecule C was complete from residue 5 to 196. Molecule B comprised residues 6 to 196 with the exception of Asp97, for which no interpretable density was observed. Refinement statistics are presented in Table 4.1.

Overall, the fit to the electron density is excellent and the structure conforms to the expected protein geometry. The final \( R/R_{\text{free}} \) values are 0.192/0.242 with 92% of the residues in the most favoured region of the Ramachandran plot (Ramachandran et al., 1963) and none in disallowed regions. Lys153 from all four molecules is in the generously allowed region. It is located in the polypeptide loop between the \( \alpha_4 \) helix and the \( \beta_9 \) strand and forms a hydrogen bond between its carbonyl oxygen and the sidechain nitrogen of Asn172, which is located in the polypeptide loop between strands \( \beta_{10} \) and \( \beta_{11} \). This hydrogen bond stabilizes the two loops. All mainchain and sidechain parameters were within or better than the expected ranges using PROCHECK (Laskowski et al., 1993).
<table>
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<th>sLe\textsuperscript{x} complex</th>
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<td>Cell angles (°)</td>
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<td>90.0, 101.9, 90.0</td>
</tr>
</tbody>
</table>

| Data collection | | |
|----------------|----------------|
| Resolution range (Å) * | 50 - 1.7 (1.76 – 1.7) | 50 - 1.6 (1.69 – 1.6) |
| Data collection temperature (K) | 100 | 113 |
| Unique Reflections | 91565 | 28401 |
| Completeness (%) * | 96.4 (95.3) | 91.3 (79.5) |
| Mosaicity (°) | 0.3 | 0.67 |
| Rmerge (%) * | 2.8 (38.4) | 4.5 (9.7) |
| I/σ\textsubscript{I} * | 15.0 (3.0) | 21.1 (7.0) |

| Refinement | | |
| Resolution range (Å) | 32.5 – 1.7 | 20.4 – 1.6 |
| R/R\textsubscript{free} | 0.192 / 0.242 | 0.212 / 0.233 |
| Protein atoms (mean B value (Å\textsuperscript{2})) | Mol A-D: 6329 (23.6) | Mol A: 1572 (15.9) |
| Water molecules (mean B value (Å\textsuperscript{2})) | 741 (27.9) | 167 (20.5) |
| Ligand atoms (mean B value (Å\textsuperscript{2})) | 4 phosphate 20 (38.4) | 1 sLe\textsuperscript{x} 55 (13.6) |
| 1 citrate 13 (23.2) |

| rms deviations from standard values | | |
| bond lengths (Å) | 0.014 | 0.009 |
| bond angles (°) | 1.45 | 1.34 |

| Ramachandran plot | | |
| % Residues in most favored regions | 92.0 | 92.2 |
| Outliers | - | - |

Table 4.1 - Refinement statistics

* Values in parentheses are for the outermost resolution shell, 1.70-1.78 Å for native SSL11-US6610 and 1.60-1.69 Å for the SSL11-US6610:sLe\textsuperscript{x} complex.
4.5 The monomer structure of SSL11-US6610

SSL11 has the classical two-domain fold that is shared by the bacterial SAgs and other SSLs (figure 4.2A). The N-terminal domain (residues 22-88) forms an OB fold comprising strands β1 to β5, which have a shear number of S=8 (see figure 1.7). The C-terminal domain (residues 98-196) forms a β-grasp fold in which a five-stranded mixed β-sheet (β7-β6-β12-β9-β10) is packed around the conserved amphipathic helix α4. The topology of the two domains is shown in figure 1.6C.

A single phosphate ion is bound to each SSL11 molecule at a site on helix α4 of the C-terminal domain. In each molecule, the phosphate is bound by residues His140 and Asp143. The phosphates bound to SSL11 molecules B and C have an additional intermolecular interaction made by Lys144 from a neighboring SSL11 molecule (figures 4.2B-C). Each phosphate ion also forms hydrogen bonds with between one and three water molecules, of which one forms a water bridge with the peptide amide of the nearby Lys136.

Pair-wise superpositions of the four molecules of SSL11-US6610 shows high similarity with an rmsd in Cα positions for 186-189 residues of between 0.44 Å and 0.98 Å (table 4.2). The main region of variation between the four molecules is in the β6-β7 strands (figure 4.3 in yellow), where dimerisation occurs (discussed in section 4.6). The four molecules can be grouped into two similar pairs; molecules A/D and molecules B/C. Molecules A and D share three regions of distinct difference when compared to molecules B and C (figure 4.3 in red). In addition, molecule D has two regions of difference to the other three molecules (figure 4.3 in blue); the loop region between β strands β4 and β5, and at the C-terminal end of the β10 strand.
Figure 4.2 - The crystal structure of SSL11-US6610
A) Ribbon diagram and amino acid sequence of SSL11-US6610, with secondary structures labeled. B) and C) Phosphate binding to SSL11-US6610. The protein structure is shown as a ribbon diagram and the phosphate ion in ball and stick mode. Hydrogen bonds are shown with broken lines and distances are in Å. Water molecules are drawn as yellow spheres. Molecule A is in yellow and molecule C is in green. The electron density for the phosphate ion is from a 2Fo-Fc map and is contoured at 1.0σ.
The three-dimensional structure of SSL11-US6610 is more similar to SSL5 than SSL7, consistent with their greater sequence identity (42% identity with SSL5 compared to 33% identity with SSL7). Superposition of the four molecules of SSL11-US6610 onto SSL5 gives an rmsd in Cα positions of between 0.88 Å and 1.06 Å for 179-184 residues, compared with values of between 1.59 Å and 1.70 Å for SSL7, based on the Cα positions for 170-177 residues (table 4.2).

<table>
<thead>
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<th>B</th>
<th>C</th>
<th>D</th>
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<td>170 / 1.59</td>
<td>187 / 0.64</td>
<td>186 / 0.98</td>
</tr>
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</table>

Table 4.2 - Similarity between the three-dimensional structures of SSL11-US6610, SSL5 and SSL7

Individual molecules (A-D) of SSL11-US6610 were superimposed on each other and to the published structures of SSL5 and SSL7 (Al-Shangiti et al., 2004; Arcus et al., 2002) in a pair-wise basis. The results are presented as number of residues used / rmsd for the Cα atoms (Å).

Figure 4.3 – Structural variation between the four molecules of SSL11-US6610

Superposition of the four molecules (A-D) of SSL11-US6610. Green indicates regions of high similarity, red indicates variation between molecules A/D and B/C, blue indicates variation observed in molecule D, and yellow indicates variation between all four molecules.
4.6 Dimerisation of SSL11-US6610

Two putative dimers are formed by the four molecules in the asymmetric unit – A/B and C/D. In the A/B dimer, seven hydrogen bonds are formed between the β7 strands of the monomers; six are between the peptide carbonyl and amide groups from His112 to Ser118 and one is between the His112 sidechain and the Ser118 carbonyl group (figure 4.4A). The bond lengths are between 2.7 and 3.0 Å. In the C/D dimer, only the six hydrogen bonds between the peptide chains are observed, due to a different rotamer conformation of His112. Slight differences in the orientation of the β6 and β7 strands results in a difference in the angle at the dimer interface of ~7° between the A/B and C/D dimers (figure 4.4B). This shift also places the His112 sidechain of molecule D approximately 1 Å further from the Ser118 carbonyl group of molecule C, in the C/D dimer, such that no hydrogen bond is formed. The crystal structure of SSL5 reveals a similar dimer formation, consisting of six hydrogen bonds between the β7 strands (Arcus et al., 2002) (figure 1.11B). This is in contrast to SSL7, which forms a β-sandwich across the β-strands β6, β7, β9 and β12 (figure 1.11C).

Dimer formation creates a continuous 10-stranded β-sheet which extends through both C-terminal domains and forms a saddle-like groove. The widest point in this groove is 25.5 Å in the A/B dimer and 23 Å in the C/D dimer, which is large enough to accommodate a strand of double-helix DNA (22-24 Å wide) as previously suggested for SSL5 (Arcus et al., 2002). However, unlike the highly positively-charged saddle-like groove of SSL5, the SSL11 equivalent is of mixed charge. The buried surface area of the dimer interface of SSL11-US6610 is small (182.3 Å² in both dimer forms), compared to 287 Å² in SSL5 and 1122 Å² and 1146 Å² from two crystal forms of SSL7.
Figure 4.4 - Dimerisation of SSL11
A) Ribbon and stick diagram showing the hydrogen bonding between SSL11-US6610 molecules A and B at the dimer interface. Distances are given in Å. B) Ribbon diagram of the two SSL11-US6610 dimers formed in the crystal, where the A/B dimer is in green and the C/D dimer is in red. The angles are calculated between Asn163, His112 of one molecule (A or C) and Asn163 of the other molecule (B or D).
4.7 Allelic variation in SSL11

In related proteins with conserved function, active sites and binding sites are expected to contain conserved or similar residues. Because of the high variability between SSL11 alleles (figure 3.3B), a structural map of the variation was created (figure 4.5) to identify regions that are potentially important for function. A high degree of conservation is observed in the β10 strand and the following extended loop region as well as in the α4 helix. Many of the other conserved residues are involved in intra-molecular interactions, such as the internal residues of the β4 and β8 strands, and the α3 helix. The β7 strand, which is involved in dimer formation in SSL11-US6610, is not conserved however, suggesting that other alleles may dimerise differently, if at all.

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**Figure 4.5 - Structural conservation of SSL11**

A ribbon diagram of SSL11-US6610 coloured to represent conservation in the amino acid sequence between SSL11 alleles. Residues that are fully conserved in the eight sequenced alleles are in red. Positions where two types of residues are found are coloured blue. Variable residues, where more than two different types of amino acids are found, are coloured green.
4.8 Attempted co-crystallization of SSL11-US6610 with Sia or FcαRI

Attempts were made to prepare diffraction-quality crystals of SSL11-US6610 in complex with either Sia or FcαRI (see section 2.2.3 i). Precipitation occurred in all conditions tested with Sia and in 366 of 384 conditions tested with FcαRI. Therefore, these attempts were discontinued.

4.9 Sialyl Lewis X (sLe³)

The blood group antigen, sLe³, is a tetrasaccharide found on P-selectin glycoprotein ligand-1 (PSGL-1) and has an important role in leukocyte rolling (see section 1.3.3 i) (Goetz et al., 1997). It comprises a Sia, galactose (Gal), fucose (Fuc) and N-acetyl glucosamine (GlcNAc). The chemical structure is Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc and is shown in figure 4.6.

Figure 4.6 - Chemical structure of sLe³

The chemical structure of sLe³ with the carbon atoms numbered for the individual sugars.
4.10  Crystallization of SSL11-US6610 in complex with sLe\(^\uparrow\)

The quality of purified SSL11-US6610 for crystallization trials was assessed by dynamic light scattering and gave a Cp/Rh of 17.2%. Crystals formed in 33 of the 480 conditions (see section 2.2.3 i) over three weeks and several of these conditions were fine-screened for crystal optimization. Large polyhedral crystals (>0.2 x 0.1 x 0.1 mm) were grown in sitting drops of 0.5 \(\mu\)L protein (15.8 mg/mL in 20mM Tris-HCl pH 7.4), 0.5 \(\mu\)L 10 mM sLe\(^\uparrow\), and 1 \(\mu\)L 20% PEG 3350, 0.2 M tripotassium citrate at 18 C\(^\circ\) (figure 4.7).

![Figure 4.7 - Crystals of the SSL11-US6610:sLe\(^\uparrow\) complex](image)

Two orthogonal views of crystals of SSL11-US6610 in complex with sLe\(^\uparrow\). The crystals are polyhedral in shape and were grown in identical conditions (20% PEG 3350, 0.2M tripotassium citrate).

4.11  Determination and refinement of the SSL11-US6610:sLe\(^\uparrow\) crystal complex

The crystals were monoclinic, space group C2, with unit cell dimensions a = 96.70 Å, b = 57.79 Å, c = 43.40 Å, \(\beta = 101.9^\circ\), and a Matthews coefficient of 2.47 Å\(^3\) Da\(^{-1}\) for one SSL11-US6610:sLe\(^\uparrow\) complex per asymmetric unit with 50.3% solvent content.

Data to 1.44 Å resolution were collected from a single crystal, at the University of Otago, New Zealand. Due to incompleteness in the highest resolution shells however, the data set was truncated to 1.6 Å (refinement statistics presented in table 4.1). The structure was solved by molecular replacement using PHASER (McCoy et al., 2005), using the coordinates of the native SSL11-US6610 molecule C as a search model. After cycles of manual rebuilding using COOT (Emsley and Cowtan, 2004) and refinement using Refmac5 (Murshudov et al., 1997), the final model comprised residues 5 to 72 and 82 to 196 for SSL11, with no interpretable
density found for residues 73-81. A large continuous piece of electron density was located near the $\beta_{10}$ and $\beta_{11}$ strands, into which sLe$^x$ was modeled. Additionally, a small piece of electron density was observed nearby, into which a citrate molecule was modeled.

Overall, the fit to the electron density is excellent and the structure conforms to the expected protein geometry. The final $R/R_{\text{free}}$ values are 0.212/0.233 with 92.2% of the residues in the most favoured region of the Ramachandran plot (Ramachandran et al., 1963) and none in disallowed regions. As in the native structure of SSL11-US6610, Lys153 is in the generously allowed region and again forms a hydrogen bond between its carbonyl group and Asn172. All mainchain and sidechain parameters were within or better than the expected ranges using PROCHECK (Laskowski et al., 1993).

4.12 The crystal structure of SSL11-US6610:sLe$^x$

A single molecule of SSL11-US6610 was refined, lacking the $\beta$-strand $\beta_{5}$ and the preceding polypeptide loop (residues 73 to 81). One sLe$^x$ molecule and one citrate ion were included in the model, both of them interacting with residues in the C-terminal $\beta$-grasp domain (figure 4.8). The citrate forms two hydrogen bonds with Arg158 and a single water bridge to Lys156 (figure 4.8B). The citrate also hydrogen bonds with seven water molecules with distances between 2.50 and 3.07 Å.

Superposition of the protein molecule from the SSL11-US6610:sLe$^x$ complex onto the native monomers of SSL11-US6610 indicates a good overall fit (table 4.3 and figure 4.9). As observed for molecules A-D in the native structure, there is variation in the $\beta_{6}$-$\beta_{7}$ region. In addition, the polypeptide between the $\alpha_{2}$ helix and $\beta$-strand $\beta_{1}$ shows variation compared to the other monomers. The $\beta$-strand $\beta_{1a}$ and the $\beta_{2}$-$\beta_{3}$ hairpin are more similar to molecules A and D, while the polypeptide connecting the two domains is most similar to molecules B and C.
Figure 4.8 - The citrate and sLe^a binding sites of SSL11-US6610

A) Ribbon diagram of SSL11 showing the location of sLe^a (yellow sticks) and citrate (orange sticks) binding to the C-terminal β-grasp domain. B) Close-up view of the citrate binding site. The electron density for the citrate ion is from a 2F_o-F_c map and is contoured at 1.2σ. Water molecules are drawn as yellow spheres, hydrogen bonds are drawn as broken lines and distances are in Å. C) Close-up view of the sLe^a binding site involving the β10 strand and the region between the β10 and β11 strands.
Superposition of the SSL11 molecule from the SSL11-US6610:sLe\(^x\) complex onto SSL5 and SSL7 gives similar rmsd values for a comparable number of residues, when compared with the native SSL11-US6610 molecules (table 4.3).

<table>
<thead>
<tr>
<th>Molecule</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>SSL5</th>
<th>SSL7</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSL11-US6610:sLe(^x)</td>
<td>181 / 0.86</td>
<td>180 / 0.80</td>
<td>182 / 0.75</td>
<td>181 / 1.00</td>
<td>176 / 1.04</td>
<td>168 / 1.70</td>
</tr>
</tbody>
</table>

Table 4.3 - Similarity between the structure of SSL11-US6610 from the sLe\(^x\) complex, with SSL11-US6610 from the native structure, SSL5 and SSL7.

The SSL11 monomer from the SSL11-US6610:sLe\(^x\) complex was superimposed onto the individual molecules of SSL11-US6610 (A-D) from the native structure and the published structures of SSL5 and SSL7 (Al-Shangiti et al., 2004; Arcus et al., 2002). The results are presented as number of residues used / rmsd for the C\(\alpha\) atoms (Å).

Figure 4.9 - Superposition of the SSL11 molecules

Superposition of the SSL11 molecule from the SSL11-US6610:sLe\(^x\)-complex onto the four molecules of SSL11-US6610 (A-D) from the native structure. Regions of the monomer from the SSL11-US6610:sLe\(^x\)-complex that vary from molecules A-D are in red. Regions that are more similar to molecules A and D are in blue and regions that are more similar to molecules B and C are in yellow. Regions of high similarity are in green.
4.13 The sLe\(^\chi\) binding site

The sLe\(^\chi\)-binding site of SSL11-US6610 is in the C-terminal \(\beta\)-grasp domain, in a V-shaped depression formed between the \(\beta_{10}\) strand and the loop region leading into the \(\beta_{11}\) strand (figure 4.8C). No gross structural changes occur in the binding site between the bound and unbound state (figure 4.10), indicating that sLe\(^\chi\) binds to a pre-formed site (rmsd of 0.24 Å for C\(\alpha\) positions of residues 164-186).

The negatively-charged sLe\(^\chi\) binds to a region of high positive charge on SSL11-US6610 (figure 4.11). The conformation of sLe\(^\chi\) is stabilized by three water bridges and an internal hydrogen bond. Two water bridges are between Sia and Gal, and one is between GlcNAc and Fuc. The internal hydrogen bond is between O8 on the glycerol sidechain of Sia and O1A on the carboxyl sidechain of Sia. The sLe\(^\chi\) molecule moulds to the surface of SSL11-US6610 in a conformation (rmsd of 0.62 Å for all atoms) that is highly similar to that observed in the crystal structures of sLe\(^\chi\) in complex with E- and P- selectin (Somers et al., 2000).
Figure 4.11 - Interaction of ligands with the surface of SSL11

Image of the interaction between sLe\(^\alpha\) (yellow sticks) and the charged surface of SSL11-US6610. A) looking onto the surface of SSL11-US6610 and B) looking across the surface of SSL11-US6610. Broken lines represent hydrogen bonds to water molecules (yellow spheres). The electrostatic potential of the SSL11 surface was calculated with PyMOL and ranges from blue (positive) to red (negative). The electron density for sLe\(^\alpha\) is from a 2F\(_{o}\)-F\(_{c}\) map and is contoured to 2.3\(\sigma\).
The region of SSL11 to which sLe\textsuperscript{x} binds, is 8-10 amino acids shorter in SSLs and TSST-1 compared with SAgs. The shortened loop forms a “V” shape in which sLe\textsuperscript{x} sits. A total of 29 hydrogen bonds are formed by sLe\textsuperscript{x} – ten to protein atoms of SSL11 and 19 to 16 water molecules (figure 4.11). Seven residues bind directly to sLe\textsuperscript{x} - Phe166, Thr168, Glu170, Gln176, His178, Arg179 and Asp182. Hydrogen bonding occurs with the sidechains of these seven residues, except for Phe166, which binds through its carbonyl oxygen.

The disaccharide Sia-Gal contributes to eight of the ten hydrogen bonds between sLe\textsuperscript{x} and SSL11 protein atoms. Sia makes a total of ten hydrogen bonds; six to SSL11 and four to water molecules (figure 4.12A). The hydroxyl group forms a single hydrogen bond with a water molecule and the N-acetyl nitrogen hydrogen bonds to the peptide carbonyl group of Phe166. The carboxyl group of Sia makes one hydrogen bond to a water molecule and two hydrogen bonds to Thr168; one to its peptide amide group and one to the sidechain oxygen. The glycerol sidechain forms the most extensive interactions, with two hydrogen bonds to Arg179, one to Asp182 and two to water molecules.

Gal makes a total of six hydrogen bonds; two to SSL11 and four to water molecules (figure 4.12B). The two hydroxyl groups of Gal coordinate three water molecules between them. The hydroxymethyl group of Gal forms hydrogen bonds to Glu170 and Gln176 in addition to a single water molecule.

GlcNAc makes a total of six hydrogen bonds; one to SSL11 and five to water molecules (figure 4.12C). The N-acetyl group binds to four waters, while the hydroxymethyl group binds to a single water molecule and to the sidechain of His178. Fuc makes a total of seven hydrogen bonds (figure 4.12D). The three hydroxyl groups between them bind to six water molecules and the side chain of Glu170. A summary of the hydrogen bonding is presented in table 4.4.
Figure 4.12 - Hydrogen bonding of the sLe^x sugars

Hydrogen bonding (broken lines) of the individual sugars of sLe^x (yellow sticks) to SSL11-US6610 (green ribbon and sticks) and waters (yellow spheres). A) Sia, B) Gal, C) GlcNAc and D) Fuc. Distances of hydrogen bonds are in Å.
<table>
<thead>
<tr>
<th>Sugar</th>
<th>Atom</th>
<th>Hydrogen bond formed to</th>
</tr>
</thead>
<tbody>
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<td>Sia</td>
<td>O1α</td>
<td>Thr168 N</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sia O8 #</td>
</tr>
<tr>
<td></td>
<td>O1β</td>
<td>Thr168 Oγ1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H2O 42★</td>
</tr>
<tr>
<td></td>
<td>O4</td>
<td>H2O 39</td>
</tr>
<tr>
<td></td>
<td>N5</td>
<td>Phe166 O</td>
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<tr>
<td></td>
<td>O7</td>
<td>H2O 16★</td>
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<tr>
<td></td>
<td></td>
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</tr>
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<td></td>
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<td>Arg179 Nη2</td>
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<td>O9</td>
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<td>Asp182 Oδ2</td>
</tr>
<tr>
<td>Gal</td>
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<td>H2O 16★</td>
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<tr>
<td></td>
<td>O4</td>
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<td>Fuc</td>
<td>O2</td>
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<td>O3</td>
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<td></td>
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<td>H2O 91</td>
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</tr>
<tr>
<td></td>
<td>O5</td>
<td>H2O 315</td>
</tr>
<tr>
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</tr>
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<td>H2O 55★</td>
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<td>O6</td>
<td>His178 Nε2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H2O 12</td>
</tr>
</tbody>
</table>

Table 4.4 - Summary of hydrogen bonds formed by sLe^x
★ indicates water molecules involved in forming water bridges and # indicates internal hydrogen bonds.

### 4.14 The SSL11:sLe^x dimer

The SSL11 molecule in the SSL11-US6610:sLe^x complex dimerises with a symmetry-related molecule in the crystal structure. The crystal dimer is very similar to the non-crystallographic dimers seen in the native SSL11-US6610 crystal. Six hydrogen bonds between 2.80 and 2.83 Å in length are formed along the β7 strand, between the peptide carbonyl and amide groups of residues 112-118 (figure 4.13A). Compared with the two dimers formed in the native crystal, the dimer formed by SSL11 in the SSL11-US6610:sLe^x complex creates a much
wider saddle-shaped interface, about 22° wider than the C/D dimer (figure 4.13B). In this
dimeric structure, the opposing sLe³ binding sites are separated by ~42 Å (figure 4.13C).

**Figure 4.13 - The SSL11 dimer in the crystal structure of SSL11:sLe³**

A) A close-up view of the dimer interface formed by SSL11 in the SSL11-US6610:sLe³ complex. Hydrogen bonds are shown by broken lines with distances in Å. B) The angle between SSL11 monomers varies between SSL11 from the SSL11-US6610:sLe³ dimer (green) and the C/D dimer from the native data set (red). C) Ribbon diagram looking into the saddle-shaped dimer, showing the location of the two sLe³ molecules (yellow sticks).
4.15 Comparison of sLe\(^x\) binding site with other SSLs

The amino acid sequences of SSLs 1-14 from the MW2 strain (Kuroda et al., 2001) were aligned with the alleles of SSL11 (see figure 3.3A) using Clustal W (Thompson et al., 1994) and the residues that form the sLe\(^x\) binding site of SSL11 were compared (figure 4.14). SSLs 3 and 6 share all of the residues whose side chains bind directly to sLe\(^x\) in the SSL11-US6610:sLe\(^x\) complex structure. SSLs 2, 4 and 5 have all the conserved residues except the histidine (His178 in SSL11-US6610). This forms a single hydrogen bond to the hydroxymethyl oxygen of the GlcNAc from sLe\(^x\). Modeling of an Asp or Asn as found in SSLs 2, 4 and 5 into this position in the SSL1-US6610:sLe\(^x\) structure indicates no adverse effects that might inhibit sLe\(^x\) binding to these proteins. Therefore, it is highly likely that in addition to all identified alleles of SSL11, SSLs 2-6 will all be able to bind sLe\(^x\).

| SSL11-1     | RKHLIDKHDLYKTEPK-DSKIRITMKNGGYTFELNKLQPHRMGDTIDSRNIEKIEVNL---- |
| SSL11-2*    | RKHLIDKHDLYKTEPK-DSKIRVTMKNGDFYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| SSL11-3     | RKHLIDNGNLYNTEPK-DSKIKVTMDDGGFYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| SSL11-4     | RKHLIDKHDLYKTEPK-DSKIRITMKNGGYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| SSL11-5     | RKHLIDKHDLYKTEPK-DSKIRVTMKNGGYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| SSL11-6     | RKHLIDKHDLYKTEPK-DSKIRVTMKNGGYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| SSL11-7     | RKHLIDKHDLYKTEPK-DSKIRITMKNGGYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| SSL11-8     | RKHLIDKHDLYKTEPK-DSKIRITMKNGGYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| MW2-SSL5    | RQYLIQNFDLYKFFPK-DSKIVMKDDGYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| MW2-SSL3    | RKHLIEKHLNYGNNMS--GTIVIKMNGGYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| MW2-SSL4    | RKHLIEKHLNYGNNMS--GTIVIKMNGGYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| MW2-SSL2    | RKHLIEKHLNYGNNMS--GTIVIKMNGGYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| MW2-SSL6    | RQXLKMKKQNGDLKDGOKTKYTFELNKLQTHGVIDGRNIEKIEVNL---- |
| MW2-SSL1    | RQHLVKNYGLYKTSSK-DGQRSHSLDGQSYNLDSLKTDFKLMFKMGIEVIESQIKIDVQK--- |
| MW2-SSL13   | RQHLISQGSLGLXGK--GQGYTITMDGTHHIDLSQKLEKRCYGESIDGRQIKILVENK-- |
| MW2-SSL14   | RQHLIKNKKNLGEGFY--GQGQITMDGTHHIDLSQKLQLEKRCYGESIDGRQIKLVMK--- |
| MW2-SSL11   | RQHLIKNKKNLGEGFY--GQGQITMDGTHHIDLSQKLEKRCYGESIDGRQIKLVMK--- |
| MW2-SSL10   | RQHLIKNKKNLGEGFY--GQGQITMDGTHHIDLSQKLEKRCYGESIDGRQIKLVMK--- |

Figure 4.14 - Amino acid sequence alignment of the SSLs

The amino acid sequences for SSLs 1-14 from the MW2 strain were aligned with the previously identified alleles of SSL11 previously identified (see figure 3.2). Alleles of SSL11 are at the top and residues that have been identified in sLe\(^x\) binding are highlighted in blue. * indicates SSL11-US6610.

Besteboer and colleagues (2006) showed that SSL5 bound PSGL-1 by a Sia-dependent mechanism. Furthermore, structural superposition of the region encompassing the sLe\(^x\)-binding site in SSL11 (residues 164-186) onto the equivalent residues in SSL5 (residues 171-193) gives an rmsd of 0.40 Å for C\(^\alpha\) atoms (figure 4.15). This suggests that SSL5 should bind to the sLe\(^x\) moiety of PSGL-1.
Figure 4.15 - SSL5 can potentially bind sLeα
Superposition of SSL5 (red) onto the sLeα-binding site of SSL11-US6610 (green). The sLeα molecule is drawn as black lines. The coordinates for SSL5 are from the pdb file 1M4V (Arcus et al., 2002).
4.16 Flow cytometric analysis of SSL11 competition with an anti-sLe\(^x\) mAb

The ability of SSL11 to compete for binding with a mAb against sLe\(^x\) (KM93) on neutrophils was tested by flow cytometry through a Becton Dickinson FACScan. Neutrophils were checked for neuraminidase-sensitive binding of KM93 (Figure 4.16A). SSL11-GL10 competed for binding with KM93 in untreated neutrophils (Figure 4.16B), while no difference in staining was observed with cells treated with neuraminidase (figure 4.16C). Previous experiments showed that FITC-labeled SSL11 stains neutrophils brightly. Staining of neutrophils with SSL11-US6610-FITC was reduced to control levels, when cells were treated with neuraminidase (figure 4.16D).

Figure 4.16 - Inhibition of anti-sLe\(^x\) mAb binding to neutrophils by SSL11-GL10

A) Histogram showing the binding of KM93 mAb to untreated (-) or neuraminidase-treated (+) neutrophils. Histograms showing the dose-dependent effects on staining with KM93 by addition of SSL11-GL10 in untreated (B) and neuraminidase-treated (C) neutrophils. SSL11-GL10 was added to samples as indicated: 0 = 0 μg, a = 3.125 μg, b = 6.25 μg, c = 12.5 μg, d = 25 μg, e = 50 μg, f = 100 μg. D) Histogram showing binding of SSL11-US6610-FITC to untreated (-) and neuraminidase-treated (+) neutrophils.
4.17 Kinetic analysis of SSL11 binding to sLe\(^x\)

The \(K_D\) values for SSL11-GL10 and SSL11-US6610 binding to sLe\(^x\) were determined by steady state affinity using biosensor analysis (figure 4.17), as performed in section 3.9. The calculated \(K_D\) for SSL11-GL10 binding to sLe\(^x\) was \(25.4 \pm 4.6 \times 10^{-6}\) M with a Hill coefficient of \(0.73 \pm 0.02\). Likewise, the calculated \(K_D\) for SSL11-US6610 binding to sLe\(^x\) was \(0.85 \pm 0.09 \times 10^{-6}\) M with a Hill coefficient of \(0.73 \pm 0.04\) (all values ± SEM).

Figure 4.17 - Steady state curves of SSL11 binding to sLe\(^x\)

Various concentrations (16 \(\mu\)M, 8 \(\mu\)M, 4 \(\mu\)M, 2 \(\mu\)M, 1 \(\mu\)M, 0.5 \(\mu\)M, 0.25 \(\mu\)M, 0.125 \(\mu\)M) of A) SSL11-GL10 and B) SSL11-US6610 were injected in triplicate over a sLe\(^x\) surface. The values at Req for both alleles of SSL11 are shown on a single plot standardised by fraction bound, where \(B_{max}\) is \(y=1\) and the lines indicate model fitting to the Hill equation.
4.18 Competition binding studies with FcαRI and sLe\(^\text{x}\)

SSL11-US6610 was removed from an FcαRI-coated biosensor surface by an injection of 1 mM sLe\(^\text{x}\) in HBS-EP running buffer (figure 4.18). This was in contrast to the corresponding studies with Sia, in which Sia was only able to regenerate the surface in water but not in buffer (see section 3.10). This was indicative of a stronger inhibition by sLe\(^\text{x}\), suggesting that the binding sites on SSL11 for FcαRI and sLe\(^\text{x}\) are either identical or closely linked.

Figure 4.18 - SLe\(^\text{x}\) regeneration of an FcαRI surface
Sensorgram showing the removal of SSL11-US6610 from an FcαRI surface by injection of 1 mM sLe\(^\text{x}\) in HBS-EP running buffer.
4.19 Discussion

SSL11-US6610 is structurally homologous to the bacterial SAgs and other SSLs. Like SSL5 and SSL7, SSL11-US6610 is present as a monomer in solution but forms a putative homodimer in the crystal structure. The fact that SSL11-US6610 formed similar homodimers in two different crystal forms with different crystal packing suggests that the dimer is not an artifact of crystal packing. Rather, it suggests that this is a preferred mode of interaction that occurs at higher concentrations of SSL11-US6610. The dimer is very similar to that formed by SSL5, with which it shares 42% amino acid sequence identity (MW2 strain), the highest out of all the SSLs (Baba et al., 2002). Both SSL5 and SSL11-US6610 dimerise through the antiparallel alignment of their respective \( \beta \)-strands, which form a number of hydrogen bonds. Four of the hydrogen bonds in the SSL5 dimer are between the peptide amide and carbonyl groups and two are between the carbonyl oxygen of Ala119 and a sidechain nitrogen of His124 across both molecules. For SSL11-US6610, six hydrogen bonds are formed between the peptide amide and carbonyl groups of His112 to Ser118. In the A/B dimer, a seventh hydrogen bond is formed between the His112 sidechain and the Ser118 carbonyl group.

When comparing the three SSL11-US6610 homodimers, a considerable degree of flexibility in the dimer interface is observed. It is possible that this hinge action may allow the dimer to bind ligands in the saddle-shaped region, or allow some freedom in conformation for crosslinking receptors, such as PSGL-1. Both alleles of SSL11 have a Hill coefficient (nH) of 0.72 when binding BSA-sLe\(^x\), suggesting that at least in the biosensor model, bound SSL11 affects further binding by other SSL11 molecules in a negative manner. This data argues against the possibility of receptor crosslinking by SSL11 dimers, but it does not accurately model the interactions that can occur on the cell surface. Firstly, in the biosensor model, 3-4 molecules of sLe\(^x\) are coupled to each molecule of immobilized BSA by a 14-atom spacer (Dextra Laboratories, UK). Therefore, the distance between two molecules of sLe\(^x\) linked to the same BSA molecule may not be great enough to accommodate SSL11 dimer formation. Second, individual molecules of BSA-sLe\(^x\) may be too far separated to allow for crosslinking by SSL11. Receptors are typically mobile along the cell surface. Initial binding of SSL11 monomers to membrane-bound receptors would increase the local concentration of SSL11 and may promote dimerisation and receptor crosslinking.
SLEx binds to SSL11-US6610 in the same conformation as it does to P- and E-selectin. Unlike the selectins, which bind primarily to Fuc (Somers et al., 2000), SSL11-US6610 only makes a single hydrogen bond to Fuc. In addition, this interaction is not Ca\(^{2+}\)-dependent for SSL11 as it is for selectins. The primary interaction between SSL11 and sLe\(^\alpha\) is with the disaccharide Sia-Gal, which contributes to eight of the ten hydrogen bonds formed between sLe\(^\alpha\) and protein atoms. In the SSL11-US6610:sLe\(^\alpha\) complex structure, the conformation of sLe\(^\alpha\) is supported by the formation of three water bridges, of which one (Fuc to GlcNAc) is also present in the E-selectin:sLe\(^\alpha\) complex structure (Somers et al., 2000). Additionally, the internal hydrogen bond between Sia O8 and Sia O1\(\alpha\) is present in both the E- and P-selectin:sLe\(^\alpha\) complexes, although the distance is much shorter in SSL11-US6610:sLe\(^\alpha\) (2.72 Å compared to 3.11 Å and 3.34 Å for E- and P-selectin respectively). Although carbohydrates are inherently flexible and can adopt many conformations, only one might allow for ligand binding (Carver et al., 1989).

The sLe\(^\alpha\) binding site is created by the β10 strand and the following loop of polypeptide chain that leads into the β11 strand. This polypeptide is 8-10 amino acids shorter in the SSLs compared to SAgs, with the exceptions of TSST-1, SpeM and SpeN. The residues involved in binding to sLe\(^\alpha\) are fully conserved in all alleles of SSL11, supporting the previous assumption that although there is a high degree of sequence variation, these proteins are indeed alleles. In addition, it seems likely that FcαRI also binds to this site, as terminal Neu5Aca2-3Galβ1-4GlcNAc (sialylLacNAc) is observed in the FcαRI crystal structure (Herr et al., 2003) and sLe\(^\alpha\) was able to regenerate an immobilised-FcαRI biosensor surface. Neuraminidase treatment of neutrophils completely abolished staining by FITC-labeled SSL11, suggesting that a single, Sia-dependent-binding site is involved in cell surface binding.

The close proximity of the citrate-binding site to the sLe\(^\alpha\)-binding site may have an important and related role. PSGL-1 is the natural ligand for P-selectin and displays O-linked sLe\(^\alpha\) in close proximity to sulphated tyrosines which are imperative for binding (Wilkins et al., 1995). Therefore, the potential coordination of a sulphate anion by Arg158 and Lys156 may be an important factor in SSL11 binding to PSGL-1.
Previous results showed that there was a high degree of allelic variation in the N-terminal portion of SSL11 (section 3.2). This translates to the majority of differences being located in the OB fold, which presents a well characterised binding face (Arcus, 2002). It is possible that other alleles of SSL11 may bind additional ligands through the OB face.

SSL11-US6610 and SSL11-GL10 are identical in sequence over the region involved in binding to sLe\(^x\), but have different dissociation constants. There is a 30-fold difference between the K\(_D\) values of SSL11-GL10 and SSL-US6610 binding to sLe\(^x\), compared to a 12.5 fold difference in binding to Fc\(\alpha\)RI (table 4.5). Variation outside the binding site may explain the difference in K\(_D\) values between alleles. SSL11-US6610 has an Asp at position 165, located at the turn between \(\beta9\) and \(\beta10\), whereas the GL10 allele has a Gly. The presence of an additional Gly in the GL10 allele may give the \(\beta10\) strand additional flexibility and adversely affect the binding of sLe\(^x\). Additionally, there may be some electrostatic repulsion of SSL11-GL10 (calculated pI of 5.50 compared to 7.85 for SSL11-US6610) from the BSA which has a pI of 4.60.

<table>
<thead>
<tr>
<th></th>
<th>Fc(\alpha)RI</th>
<th>sLe(^x)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KD (M)</td>
<td>Hill Coefficient</td>
</tr>
<tr>
<td>SSL11-GL10</td>
<td>9.02 ± 1.00x10(^{-6})</td>
<td>0.93 ± 0.03</td>
</tr>
<tr>
<td>SSL11-US6610</td>
<td>0.72 ± 0.05x10(^{-6})</td>
<td>1.01 ± 0.08</td>
</tr>
</tbody>
</table>

Table 4.5 - Summary of kinetic data of SSL11

It is intriguing to speculate that a subpopulation of SSLs may share the same ability to bind sLe\(^x\). SSLs 2, 3, 4 and 11 are all expressed in the stationary phase (Fitzgerald et al., 2003; Laughton et al., 2006). Whether these proteins can interact with each other i.e. form heterodimers, is unknown. Other virulence factors of S. aureus are expressed in the stationary phase by the accessory gene regulator locus (agr), including TSST-1 (Recsei et al., 1986). In addition, S. pyogenes has a similar locus – the multiple gene activator locus (mga) – that upregulates SpeA and SpeB in stationary phase (Unnikrishnan et al., 1999). Typically, the expression and secretion of virulence proteins in the stationary phase are a response to alter environmental conditions and overcome host defense mechanisms. Expression of agr and SarA systems has been observed both in vitro and in vivo (Cheung et al., 2004).
Chapter 5 – Investigating the binding specificity of SSL11 and its effect on neutrophils

5.1 Introduction
SSL11 interacts with two important molecules of the immune system – sLe\textsuperscript{x} and Fc\alpha RI. The data suggests that SSL11 is primarily a carbohydrate-binding protein with specificity for sialated carbohydrates. The crystal structure of SSL11-US6610 in complex with sLe\textsuperscript{x} provided an insight into the molecular basis for the interaction and suggests that Neu5Ac\alpha2-3Gal is the minimum structure required for binding. Eight of ten hydrogen bonds between SSL11 and sLe\textsuperscript{x} are made to this disaccharide.

The literature clearly indicates that sLe\textsuperscript{x} is essential for leukocyte tethering, an early step in leukocyte recruitment (see section 1.3.3 i). Because leukocyte recruitment is a series of sequential processes, negative effects on tethering will affect downstream processes including adhesion, diapedesis and effective removal of pathogens. The results presented in this chapter aim to elucidate the binding specificity of SSL11 and the functional effects SSL11 has on the immune system.

5.2 Identification of SSL11 ligands
Because the carbohydrate ligand of SSL11 is likely to be expressed on other cell surface molecules (Varki, 2007), the ligands for SSL11-US6610 were identified by a pulldown assay (figure 5.1). Proteins were identified by matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) at the Australian Proteome Analysis Facility Ltd (APAF) at Macquarie University, Sydney.
Figure 5.1 - Leukocyte pulldown by SSL11-US6610
Image of a 12.5% SDS-polyacrylamide gel of proteins bound by SSL11-US6610 coupled to sepharose beads and eluted with 10 mM sialic acid, from detergent-solubilised polynuclear (lane 1) and mononuclear (lane 2) cell lysate. Lane M denotes a molecular weight standard.

Five of the nine protein bands sent for identification (bands 2-8, 10 and 11 from figure 5.1) returned with positive identification (table 5.1 – see also appendix 7.6) when submitted to the database search program Mascot (Matrix Science Ltd., London), against *Homo sapiens* entries in the swissprot database. Three of these bands (2, 3 and 8) were lactoferrin, a key molecule involved in both bacteriostasis by iron scavenging and direct bacteriostasis (see section 1.3.7). Due to differential glycosylation, lactoferrin can migrate as different bands by SDS-PAGE (van Berkel *et al.*, 1995). The other two molecules identified were CD11b (Mac-1 α chain), a highly important molecule in many processes including leukocyte recruitment and phagocytosis (see sections 1.3.3 and 1.3.5), and CD41 (platelet glycoprotein GpIIb).

No sequence was obtained for band 7 despite its obvious intensity. However, it is most likely that this protein is CD61 (GpIIIa), which forms a non-covalently associated heterodimer with CD41. The band also migrated at the expected size of CD61 of ~97 kDa. The heterodimer CD41/CD61 (or GpIIb/IIIa) is a fibrinogen-binding integrin involved in platelet adhesion and aggregation (Moroi and Jung, 1998).
Table 5.1 - Summary of hits from protein identification by MALDI-MS

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Matched to</th>
<th>Score</th>
<th>Expect</th>
<th>Mr (Da)</th>
<th>Calculated pI</th>
<th>Sequence coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Lactoferrin</td>
<td>174</td>
<td>6e-14</td>
<td>78132</td>
<td>8.50</td>
<td>63%</td>
</tr>
<tr>
<td>3</td>
<td>Lactoferrin</td>
<td>145</td>
<td>4.8e-11</td>
<td>78132</td>
<td>8.50</td>
<td>59%</td>
</tr>
<tr>
<td>4</td>
<td>CD11b (Mac-1α)</td>
<td>152</td>
<td>9.5e-12</td>
<td>127099</td>
<td>6.88</td>
<td>48%</td>
</tr>
<tr>
<td>6</td>
<td>CD41 (GpIIb)</td>
<td>146</td>
<td>3.8e-11</td>
<td>113320</td>
<td>5.21</td>
<td>44%</td>
</tr>
<tr>
<td>8</td>
<td>Lactoferrin</td>
<td>162</td>
<td>9.5e-13</td>
<td>78132</td>
<td>8.50</td>
<td>64%</td>
</tr>
</tbody>
</table>

Mononuclear and polynuclear cell proteins that bind to SSL11-US6610 were identified by MALDI-MS. The results are summarised above. Expect is a probabilistic score that estimates the likelihood that the protein identified was obtained purely by chance.

5.3 Determination of the carbohydrate specificity of SSL11

The carbohydrate-binding specificity of SSL11 was determined by the Consortium for Functional Glycomics (CFG) Core H glycan array screen v2.1. The CFG is a research initiative funded by the National Institute of General Medical Sciences (NIGMS) in the USA and is comprised of seven scientific cores formed to elucidate protein-carbohydrate interaction. At the time of processing, the glycan array consisted of 285 unique samples (see appendix 7.7). Related glycans were distributed together and numbered accordingly i.e. glycans initiating in Galβ1-3GalNAc are located at positions 115-129. Binding data for SSL11-US6610 were collected for n=6, where the highest and lowest values were removed to reduce the effect of outliers, leaving a final sample size of n=4. The relative fluorescence units (RFU) were measured for FITC-labeled SSL11-US6610 at 200, 20 and 2 μg/mL (figure 5.2).

Figure 5.2 - Glycan array v2.1

Summary graphs of results from three different concentrations of SSL11-US6610-FITC on the CFG glycan array v2.1 – A) 2 μg/mL, B) 20 μg/mL and c) 200 μg/mL. The graphs show the average fluorescence for n=4 with the standard error of the mean (+ SEM) represented by the error bars.
Table 5.2 - Summary of concentration-dependent analysis by glycan array v2.1

The glycans giving the highest mean RFU from the Glycan array v2.1 using SSL11-US6610 at 200 µg/mL were compared to the values obtained for 20 and 2 µg/mL. Highlighted rows indicate tighter binding across all concentrations tested.

SSL11-US6610 bound to a few select glycans, clustering between samples 227 and 239 with a very low overall background. As the concentration of SSL11-US6610-FITC was increased, additional ligands showed significant RFU. Although the 200 µg/mL sample showed a broader specificity for ligands than the 20 and 2 µg/mL samples, these were all greater than the maximum RFU reported for the assay, of 50,000-60,000. Therefore, a more accurate measure of specificity is to compare the glycan specificities across the three concentrations.

There were minor variations in the comparative brightness observed between the RFU for these glycans with the three concentrations of SSL11-US6610-FITC assayed (table 5.2).

<table>
<thead>
<tr>
<th>Glycan No.</th>
<th>Glycan Name</th>
<th>200 µg/ml</th>
<th>20 µg/ml</th>
<th>2 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean RFU</td>
<td>Mean RFU</td>
<td>Mean RFU</td>
<td></td>
</tr>
<tr>
<td>228</td>
<td>Neu5Aca2-3Galβ1-4(Fucα1-3)(6OSO3) GlcNAcβ –Sp8</td>
<td>53568</td>
<td>1159</td>
<td>81</td>
</tr>
<tr>
<td>229</td>
<td>Neu5Aca2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4(Fucα1-3) GlcNAcβ–Sp0</td>
<td>45413</td>
<td>39281</td>
<td>3318</td>
</tr>
<tr>
<td>230</td>
<td>Neu5Aca2-3Galβ1-4(Fucα1-3)GlcNAcβ–Sp0</td>
<td>44951</td>
<td>23529</td>
<td>2348</td>
</tr>
<tr>
<td>231</td>
<td>Neu5Aca2-3Galβ1-4(Fucα1-3)GlcNAcβ–Sp8</td>
<td>48198</td>
<td>19758</td>
<td>1714</td>
</tr>
<tr>
<td>232</td>
<td>Neu5Aca2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ–Sp8</td>
<td>46794</td>
<td>24803</td>
<td>2648</td>
</tr>
<tr>
<td>233</td>
<td>Neu5Aca2-3Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4GlcNAcβ–Sp8</td>
<td>47672</td>
<td>32598</td>
<td>4194</td>
</tr>
<tr>
<td>235</td>
<td>Neu5Aca2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-4GlcNAcβ–Sp0</td>
<td>49273</td>
<td>20718</td>
<td>1722</td>
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<tr>
<td>236</td>
<td>Neu5Aca2-3Galβ1-4GlcNAcβ–Sp0</td>
<td>42358</td>
<td>15750</td>
<td>210</td>
</tr>
<tr>
<td>237</td>
<td>Neu5Aca2-3Galβ1-4GlcNAcβ–Sp8</td>
<td>49622</td>
<td>19142</td>
<td>1537</td>
</tr>
<tr>
<td>238</td>
<td>Neu5Aca2-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ–Sp0</td>
<td>43191</td>
<td>12944</td>
<td>710</td>
</tr>
<tr>
<td>239</td>
<td>Neu5Aca2-3Galβ1-4Glcβ–Sp0</td>
<td>55739</td>
<td>39281</td>
<td>14</td>
</tr>
<tr>
<td>259</td>
<td>Neu5Gca2-3Galβ1-4(Fucα1-3)GlcNAcβ–Sp0</td>
<td>45743</td>
<td>10875</td>
<td>148</td>
</tr>
<tr>
<td>260</td>
<td>Neu5Gca2-3Galβ1-4GlcNAcβ–Sp0</td>
<td>53436</td>
<td>574</td>
<td>21</td>
</tr>
</tbody>
</table>
However, the results indicate that there was very tight specificity towards sLe\(^\alpha\)-like carbohydrates, in particular the trisaccharides Neu5A\(\alpha\)2-3Gal\(\beta\)1-3Glc (sialyllactose), and Neu5A\(\alpha\)2-3Gal\(\beta\)1-3GlcNAc (sialylLacNAc). This was consistent with the crystal structure as previously discussed, where the majority of bonding occurs to Sia and Gal (section 4.13). These data also suggest that Neu5Gc (N-glycolylneuraminic acid), another form of sialic acid (see figure 3.11), was tolerated by SSL11 albeit with lower affinity. A comparison of glycans 259 and 260 suggest that fucose has an important role in binding for Neu5Gc-linked carbohydrates.

5.4 Confirmation of the sLe\(^\alpha\)-binding site of SSL11-US6610 by site-directed mutagenesis

To confirm the predicted sLe\(^\alpha\)-binding site of SSL11-US6610, a site-directed mutant was created that targeted a sidechain critical in the interaction with sialic acid. Previous functional analyses had shown that Sia was a key component in the binding of Fc\(\alpha\)RI (section 3.11) and the SSL11-US6610:sLe\(^\alpha\) complex structure indicates that the majority of the interactions are also through the Sia (section 4.13). Therefore, the interaction with Sia was targeted. In the SSL11-US6610:sLe\(^\alpha\) crystal structure, Thr168 formed two hydrogen bonds to the carbonyl group of Sia and Arg179 formed two hydrogen bonds to the glycerol side chain of Sia. Because Arg179 made integral contacts within the structure of SSL11-US6610, it was decided that a mutant of Thr168 would be more stable. Because one of the contacts is to the peptide amide of Thr168, an alanine mutant would only remove a single hydrogen bond. Therefore, Thr168 was mutated to a proline to destroy both hydrogen bonds and provide additional steric hindrance. Thr168 has \(\varphi\) and \(\psi\) angles of -117° and 133° respectively, which are compatible with a proline substitution. This mutation was first modeled into the structure in silico using the software PyMOL (DeLano Scientific LLC) to confirm the proline would not cause any structural problems. The proline was introduced at position 168 using a two-step overlap PCR (section 2.2.1 xi). A comparable yield of soluble, recombinant protein (SSL11-T168P) was expressed using pGEX3C, compared to wild-type SSL11-US6610.
5.5 Binding studies of SSL11-T168P

To investigate the effect of the Thr to Pro mutation, binding experiments were performed by surface plasmon resonance. SSL11-T168P was injected over an FcαRI and sLe\(^x\) surface at identical concentrations to the highest previously used for the wild-type protein, SSL11-US6610. The data for SSL11-T168P were converted to a fraction bound in relation to the calculated \(B_{\text{max}}\) value for the wild-type protein on the respective surfaces (figure 5.3). Binding of the mutant was minimal for both surfaces, although the interaction with sLe\(^x\) produced a weaker dose-dependent response than the wild-type protein. It is possible that this minor response was due to a secondary binding site with a significantly lower binding affinity, or incomplete inhibition of binding by the proline mutation.

![Sensorgrams of SSL11-T168P injected over surfaces coupled with A) sLex and B) FcαRI (16 μM, 8 μM, 4 μM). C) The values at Req were converted to fraction bound using the \(B_{\text{max}}\) calculated for the wild-type protein (y=1) over the respective surfaces.](image)

**Figure 5.3 - Steady state curves of SSL11 binding FcαRI**

Sensorgrams of SSL11-T168P injected over surfaces coupled with A) sLe\(^x\) and B) FcαRI (16 μM ■, 8 μM □, 4 μM ▲). C) The values at Req were converted to fraction bound using the \(B_{\text{max}}\) calculated for the wild-type protein (y=1) over the respective surfaces.
5.6 In vitro neutrophil rolling assay

Because sLe\(^\alpha\) is vital for leukocyte tethering to endothelial cells via P-selectin, the effects of introducing SSL11-US6610 into an in vitro rolling assay was assessed at a comparable shear force (0.8 dyn/cm\(^2\)) to those exhibited in high endothelial venules. SSL11-US6610 inhibited neutrophil tethering to a P-selectin surface in a dose-dependent manner with a half maximal effect at \(~50\) nM and a maximal effect at \(75\) nM, comparable to \(22\) nM of the anti-sLe\(^\alpha\) mAb, KM93 (figure 5.4). KM93 inhibits binding of P-selectin to sLe\(^\alpha\). SSL11-T168P had no significant effect at the maximum concentration assayed (200 nM).

![Figure 5.4 - SSL11 inhibits neutrophil tethering](image)

Comparison of the number of neutrophils adhered to a P-selectin-coated microchamber after treatment with SSL11-US6610, 200 nM SSL11-T168P or 22 nM of the anti-sLe\(^\alpha\) mAb KM93, where the control (no SSL11) is represented as 100%. Error bars represent \(\pm\) SEM.
5.7 Cellular internalisation of SSL11-US6610

Flow cytometry results showed that SSL11 was able to bind to neutrophils in a Sia-dependent manner (figure 4.16D), so its cellular distribution was determined by fluorescence microscopy. SSL11-US6610 was internalised into the cytoplasm but excluded from the nucleus (figure 5.5A). Staining of the cells was rapid and evident with incubation times of 30 s on ice with SSL11-US6610-FITC. Neuraminidase treatment of the neutrophils resulted in a significant reduction in staining (figure 5.5B), consistent with the results from previous flow cytometry experiments (figure 4.16D). However, there was still some intracellular staining, which was possibly from incomplete treatment with neuraminidase.

Figure 5.5 - Neutrophils stained with SSL11-US6610-FITC
Fluorescence microscope images of adherent, (A) untreated and (B) neuraminidase-treated neutrophils stained for 5 min on ice with 0.1 μM SSL11-US6610-FITC.

Because of the high background and photo bleaching associated with FITC, the experiment was repeated using Cy5 labeled proteins. SSL11-US6610 and the Sia-binding knockout, SSL11-T168P were conjugated to Cy5 (section 2.2.2 xv) and staining of neutrophils was visualised using confocal laser scanning microscopy. Bright staining of the cytoplasm was observed with Cy5-labeled SSL11-US6610 (figure 5.6A), but not SSL11-T168P (figure 5.6B), indicating that the dim staining previously observed with neuraminidase-treated neutrophils was most likely due to incomplete treatment. Closer examination revealed that SSL11-US6610-Cy5 accumulated in brightly stained cytoplasmic structures (figure 5.6C).
Figure 5.6 - Confocal microscopy
Confocal microscope images of neutrophils stained for 1 min with (A) SSL11-US6610-Cy5 or (B) SSL11-T168P-Cy5. Cy5 staining is presented in red and DAPI (nuclear staining) is presented in blue. Panel C is the same sample as in image A, but at a greater scale of magnification to show bright vesicular staining in the cytoplasm indicated by white arrows. The image is presented in glow over, where SSL11-US6610-Cy5 labeling is in orange.
5.8 Neutrophil two-dimensional gel electrophoresis

The effect on neutrophil protein expression by the internalization of SSL11 was investigated by 2-D gel electrophoresis. A comparison between the control and SSL11-US6610-treated sample (figure 5.7) showed that areas 4, 7, 8 and 9 had an increased amount of protein in the SSL11-treated sample compared to the control. In contrast, areas 1, 2, 3 and 5, had decreased amounts of protein. Four of the protein spots that varied in intensity between the two gels were identified by QStar analysis at the Centre for Genomics and Proteomics (The University of Auckland, New Zealand) as actin variants (table 5.3 – see also appendix 7.8). Protein spots 1 and 4 were both identified as β-actin variants. Protein spots 2 and 3 were both identified as actin gamma-1. In the control sample, there is a single large spot (area 6), which is of similar intensity to spots 2 and 3 combined from the SSL11-treated sample, suggesting that the protein in spot 2 is a subpopulation which has undergone a form of modification that would reduce its pI (i.e. phosphorylation).

One of the most notable differences is the disappearance of proteins from area 2 in the control sample, and the appearance of proteins of the same molecular weight in area 4 of the SSL11-treated sample. It is most likely that these are the same proteins, but have undergone some form of modification that alters their pI. Because of their similar molecular weight and pI to protein spots 1 and 4, identified as β-actin variants, it is a possibility that the proteins within these areas as also forms of actin.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Mr(Da)</th>
<th>Calculated pI</th>
<th>Sequence coverage</th>
<th>Score</th>
<th>Queries matched</th>
<th>Matched to</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42038</td>
<td>5.29</td>
<td>25%</td>
<td>367</td>
<td>10</td>
<td>β-actin variant</td>
</tr>
<tr>
<td>2</td>
<td>29678</td>
<td>5.50</td>
<td>27%</td>
<td>316</td>
<td>7</td>
<td>ACTG-1 (actin gamma-1)</td>
</tr>
<tr>
<td>3</td>
<td>29678</td>
<td>5.50</td>
<td>24%</td>
<td>317</td>
<td>10</td>
<td>ACTG-1 (actin gamma-1)</td>
</tr>
<tr>
<td>4</td>
<td>42038</td>
<td>5.29</td>
<td>31%</td>
<td>465</td>
<td>15</td>
<td>β-actin variant</td>
</tr>
</tbody>
</table>

Table 5.3 - Summary of proteins identified by QStar

Protein spots exhibiting major differences between the control and SSL11-treated neutrophils were identified by QStar analysis.
Figure 5.7 - 2-D gel analysis of neutrophils

Whole-cell lysate of (A) untreated neutrophils and (B) neutrophils treated with 0.1 μM SSL11-US6610 were analysed by 2-D gel analysis. Areas that vary between gels are indicated by red circles. Protein spots identified by QStar are labeled in cyan.
Figure 5.7 continued
5.9 Discussion

The carbohydrates used in the CFG glycan array include various linkage groups of sialated polysaccharides, including Neu5Aca\(_2\)-3Galβ1-3, Neu5Aca\(_2\)-6Gal, Neu5Gca\(_2\)-3Galβ, Neu5Gca\(_2\)-6Galβ and sulphation of sugar moieties. SSL11-US6610 bound carbohydrate with clear specificity for the trisaccharides Neu5Aca\(_2\)-3Galβ1-4Glc (sialyllactose) and Neu5Aca\(_2\)-3Galβ1-4GlcNAc (sialylLacNAc). However, there was a preference for carbohydrates containing sialylLacNAc (including sLex) compared to sialyllactose. Although the N-acetyl group of GlcNAc forms no direct hydrogen bonds with SSL11, it interacts with four water molecules which could improve binding stability compared to Glc (section 4.13).

Binding of SSL11 to carbohydrates containing N-glycolylneuraminic acid (Neu5Gc) was also observed. The SSL11-US6610:sLe\(^x\) structure indicates that SSL11 could bind equally well to Neu5Gc as it does to Neu5Ac, as the extra OH of the N-glycolyl group (HOCH\(_2\)CO) compared to the N-acetyl (CH\(_3\)CO), would point away from SSL11. However, the glycan array data shows a significant reduction in the capacity for SSL11 to bind to carbohydrates containing Neu5Gc compared to Neu5Ac. For example, the array samples 259 and 260 are equivalent to samples 230 and 236 respectively (table 5.2), with the substitution of Neu5Gc for Neu5Ac. The binding affinity of SSL11-US6610 to Neu5Gca\(_2\)-3Galβ1-4GlcNAcβ (sample 260) is improved by the addition of a fucose (Neu5Gca\(_2\)-3Galβ1-4[Fucα1-3]GlcNAc - sample 259). Because the N-acetyl group of Sia hangs over an area of negative charge (figure 4.11), it is possible that the additional negative charge on the N-glycolyl group may cause some electrostatic repulsion. The presence of Fuc would provide additional binding stability, which would partially counter the repulsion effect by the N-glycolyl group.

The carbohydrate binding site of SSL11-US6610 was confirmed by the site-directed mutation of Thr168 to a proline. SSL11-T168P showed significantly reduced binding to sLe\(^x\) and binding to FcαRI was abolished. A lack of neutrophil staining by SSL11-T168P also confirmed previous observations by flow cytometry (section 4.16), that the carbohydrate binding site and interaction with Sia is necessary for interaction with cell surface proteins. Therefore, the functional effects of SSL11-US6610 on neutrophils are dependent on its carbohydrate-binding site.
Three ligands for SSL11 were identified by pulldown assay and subsequent MALDI-MS. These were Lactoferrin, CD41 (which forms GpIib/IIa with CD61) and CD11b (which forms Mac-1 with CD18). The results from the pulldown assay with SSL11-US6610 suggest that GpIib/IIa is the primary ligand, as CD41 was the major band. The binding of SSL11 to these proteins is most likely carbohydrate-mediated. For instance, the proposed glycan structures of lactoferrin include terminal Neu5Aca2-6Galβ1-4GlcNAc, elucidated through enzymatic reduction and gel chromatography (Matsumoto et al., 1982). However, the neuraminidase used was from Arthobacter ureafaciens which cleaves both Neu5Aca2-3 and Neu5Aca2-6 linkages (Uchida et al., 1979). Therefore, it is possible that lactoferrin actually expresses terminal Neu5Aca2-3Galβ1-4GlcNAc, to which SSL11 binds. In addition, CD41 glycans were determined to be NeuAc-Gal-GlcNAc linked to a mannose structure by fast-atom bombardment mass spectrometry (Reason et al., 1991). It is unknown whether Mac-1 expresses terminal sialyllAccNAc, sLe^x^, or related carbohydrates with which SSL11 interacts. However, the presence of Le^x^ (Galβ1-4[Fucα1-3]GlcNAc, or CD15) on neutrophil Mac-1 has been reported (Skubitz and Snook, 1987), but glycosylation of Mac-1 is cell-dependent (van Gisbergen et al., 2005).

S. aureus has been shown to present at least one lactoferrin-binding protein on its surface that recognises both human and bovine forms (Naidu et al., 1991). Surface-bound SSL11 (Laughton et al., 2006) may mediate the uptake of iron by sequestering lactoferrin. Lactoferrin binding to S. aureus was abolished when strains were cultivated on carbohydrate- and salt-rich agar. High salt (1M NaCl) has been reported to reduce the expression of the SaeRS system, which directly downregulates SSL11 expression (Novick and Jiang, 2003) (section 1.5.4 v) and SSL11 has been shown to bind carbohydrates. Therefore, a decrease in the quantity or ability of SSL11 to bind lactoferrin would be expected in these conditions.

Both of the integrin molecules, Mac-I and GpIib/IIa, have important roles in cellular adhesion. Both proteins bind fibrinogen and intercellular adhesion molecule-1 (ICAM-1); Mac-1 directly and GpIib/IIa via a fibrinogen bridge (Bombeli et al., 1998; Diamond and Springer, 1993; Simon et al., 1997; Smith et al., 1990; Wright et al., 1988). Interaction with ICAM-1 leads to stable adhesion of leukocytes to the endothelial wall, which precedes their extravasation into the site of inflammation. For platelets, interaction with ICAM-1 is an essential step in platelet-mediated clot formation (Bombeli et al., 1998).
Platelets are an important target for virulence factors. A study by Ludwig and colleagues (2004) revealed that activated platelets express PSGL-1, which is solely responsible for their aggregation with leukocytes \textit{in vitro} and correlates with increased rolling of leukocytes on cutaneous microvessels \textit{in vivo} (about one third were due to association with platelet aggregates). They also show that addition of anti-GpIIb/IIIa mAb to platelets significantly reduced their ability to roll. Therefore, SSL11 interacts with two key molecules involved in platelet aggregation and platelet-enhanced leukocyte rolling – PSGL-1 and GpIIb.

Mac-1 is expressed on most circulating monocytes and granulocytes (97\% and 90\% respectively) (Eddy \textit{et al.}, 1984). Mac-1 is involved in many important immune pathways, including leukocyte adhesion (section 1.3.3 ii and iv) and opsonophagocytosis via the complement molecule C3bi (section 1.3.5). Assuming the glycan is close to the binding / active site of Mac-1, binding of SSL11 will likely have an impact on one or more of the numerous immune functions that involve Mac-1. For instance, binding of SSL11 to CD11b may inhibit the conformational change in its C-terminal domain that allows Mac-1 to bind ICAM-1 (Xia \textit{et al.}, 2002). Additionally, neutrophil-specific glycosylation on Mac-1 allows it to modulate the function of dendritic cells (van Gisbergen \textit{et al.}, 2005).

The data showed that SSL11-US6610 had at least two effects on neutrophils which were dependent on its functional carbohydrate-binding site. First, it inhibited neutrophil rolling at nanomolar concentrations and second, the rapid internalization of SSL11-US6610 resulted in significant changes in protein expression. Neutrophils are the primary form of defense against \textit{S. aureus} infection, as it is resistant to lysis by complement and lysozyme (Foster, 2005). Therefore, the recruitment of neutrophils to sites of infection is important for clearance of \textit{S. aureus} infection. Because leukocyte recruitment is a sequential process, impairment of one step will result in inhibition of the entire process. The results showed that SSL11 was capable of direct inhibition of neutrophil rolling to a P-selectin surface. This effect may be augmented \textit{in vivo}, where an inhibition of platelet function described above, would also affect leukocyte recruitment. In addition, SSL11 selectively bound to Mac-1, which is involved in the adherence of neutrophils to the endothelial cell layer (Hentzen \textit{et al.}, 2000). Therefore, SSL11 may play a complex role in immune evasion, by modulation of leukocyte recruitment through multiple pathways.
The internalization of SSL11 was clearly dependent on its binding to the cell surface via its carbohydrate-binding site. This was supported by the abrogation of staining of neuraminidase-treated neutrophils and by the functional knockout of the Sia-binding site by site-directed mutagenesis (SSL11-T168P). SSL11 exhibited a cytoplasmic distribution with exclusion from the nucleus, making it unlikely that it would have access to bind DNA within the saddle-like groove formed by the dimer, as suggested in section 4.6. Bright, punctuate staining was observed which is indicative of vesicle formation. However, it is unknown what kind of vesicle SSL11 is localizing in (i.e. endosome, lysosome etc.). Counterstaining with vesicle-specific dyes (i.e. lyso tracker) may elucidate the type of vesicle with which SSL11 associates.

Within the cytoplasm, SSL11 mediated several changes in the neutrophil proteome. The 2-D gel analysis of neutrophils treated with SSL11-US6610 indicated that two forms of actin underwent some form of modification, which caused them to separate at a different pI or size when compared to the control sample. Modification of the actin cytoskeleton is a common approach by bacterial toxins and can occur through at least three pathways. The first is the direct modification of actin (i.e. by ADP-ribosylation), the second is the modulation of proteins that regulate the actin cytoskeleton, and the third is the increase the intracellular cyclic AMP levels by adenylate cyclases which results in actin reorganization (reviewed by Barbieri et al., 2002). Monomeric actin (G-actin, or globular actin) polymerises into microfilaments called F-actin (filamentous actin). In its monomeric state, G-actin associates with adenosine triphosphate (ATP). In F-actin polymers, the ATP is hydrolysed into adenosine diphosphate (ADP), which remains bound to the actin, and inorganic phosphate, which is released into solution (Straub and Feuer, 1989). Therefore, actin polymerization is inhibited when G-actin is tightly associated with ADP, rather than ATP. Several bacterial toxins, including the C2 toxin family, mediate direct ADP-ribosylation of actin, which results in the depolymerization of F-actin (Aktories et al., 1986; Schering et al., 1988).

The actin cytoskeleton is a vital part of cellular function and is inherently involved in many subjects discussed within this thesis. For instance, the cytoplasmic domain of PSGL-1 must be attached to actin for the functional rolling of leukocytes (Snapp et al., 2002). Inhibition of polymerization and depolymerization of actin filaments impaired integrin-mediated internalization of *S. aureus* by the epithelial cell line HEK293 (Agerer et al., 2005). In addition, remodeling of the actin cytoskeleton is necessary for the formation of pseudopodia,
which are involved in phagocytosis (Coppolino et al., 2001). Therefore, SSL11-mediated effects on actin may play an important role in pathogenesis.
Chapter 6 - Discussion

The aims of this thesis were to characterise the structure and function of SSL11. The data presented herein reveal the following key points. 1) SSL11 exhibits a high variation in amino acid sequence. 2) SSL11 is immunogenic in humans. 3) SSL11 folds in the expected classical SAg fold, comprising an N-terminal OB domain and a C-terminal β-grasp domain. 4) A functional binding site was identified by X-ray crystallography and confirmed by site-directed mutagenesis. 5) SSL11 was characterised as a Sia-dependent glycan-binding protein with specificity for Neu5Acaβ2-3Galβ1-4Glc[NAc], where [NAc] is preferred, but not necessary for binding. Residues involved in binding to sLe^x were also found to be conserved in SSLs 2-6. 6) SSL11 selectively interacts with several molecules involved in the immune system. 7) SSL11 inhibits neutrophil function and is rapidly internalized, resulting in differential protein expression.

6.1 SSL11 exhibits a high variation in primary sequence

Sequencing of ssl11 from 44 clinical isolates of S. aureus revealed three novel alleles of SSL11. A total of eight variants were identified after alignment of translated amino acid sequences with those from genome projects, which divide into two distinct clades (figure 3.2). Sequence identity of SSL11 alleles is between 56.3% and 93% (figure 3.3). The identification of a conserved carbohydrate-binding site between the alleles (section 4.15) indicates they will have some conserved function. For instance, both the GL10 and US6610 alleles bind to FcαRI and sLe^x. Although their dissociation constants differ, they behave in a similar manner, exhibiting comparable Hill coefficients for both ligands (table 4.5). However, other SSLs also share these residues involved in binding and will most likely bind to these ligands in a similar fashion. Further investigation will be required to determine if the alleles of SSL11 exhibit a function unique from the other SSLs (such as the modulation of the neutrophil proteome after its rapid internalization).
6.2 SSL11 is immunogenic

Human seroconversion to SSL11 indicates it is expressed in vivo and is immunogenic. Specific antibodies were not cross-reactive between alleles or other SSLs, as Ig titres to SSL11-US6610 were significantly different from titres to SSL11-GL10 and SSL5 (figure 3.6B). It is interesting to note that patients with acute S. aureus infection had significantly lower titres of anti-SSL antibodies than healthy donors (section 3.5), as expression of SSL11 is directly correlated with increased virulence (Gan et al., 2002; Laughton et al., 2006) and SSL7 inhibits serum-killing of bacteria (Langley et al., 2005). The presence of neutralizing antibodies against SSLs may help to protect the host from infection by S. aureus, but a larger study is required to confirm this.

6.3 Structural comparison of SSL11 with CHIPS

The structure of SSL11 comprises an N-terminal OB domain and a C-terminal β-grasp domain (section 4.5). These folds accommodate a large amount of sequence variation and therefore, a wide range of associated function. As discussed earlier (section 1.4.1 iv), CHIPS is an important immune modulator expressed by S. aureus. CHIPS forms a β-grasp domain with an α-β-α-β-α-β-α-β topology, compared to SSL11 which has a β-β-α-β-α-β-β-β topology for the equivalent domain. Superposition of CHIPS (121 residues) to SSL11-US6610 gives an rmsd in Cα positions of 1.65 Å for 64 residues (figure 6.1A).

CHIPS shares several key residues with SSL11 that are involved in Sia binding (figure 6.1B). The equivalent residues for SSL11-US6610 Phe166, Tyr167, Thr168 and Arg179 are Tyr71, Tyr72, Thr73 and Arg84 in CHIPS, respectively. SSL11-US6610 residue Asp182 is replaced by Val87, which will be unlikely to form hydrogen bonds with Sia, however it is unlikely to cause any steric hinderance. Although there are some differences in the conformation of the β-strand and side chain rotamers between CHIPS and SSL11 in the sLeX-binding site, it seems highly probable that CHIPS would also be able to interact with sialated carbohydrates. However, the glycan specificity would no doubt be different from SSL11, as the residues involved in binding to Gal, GlcNAc and Fuc of sLeX vary in CHIPS. Site directed mutagenesis of Arg84 in CHIPS resulted in impaired ability to inhibit both formyl peptide receptor (FPR1) and complement C5a receptor (CD88) activity (Haas et al., 2005). However, because the two functional activities are located on different sites, the observed effects of the Arg84 to Ala mutant were thought to be due to a conformational change.
6.4 Potential effects of glycan binding by SSL11

Neu5Acα2-3Galβ1-4GlcNAc (sialylLacNAc) was identified as the primary glycan ligand for SSL11 and is commonly expressed at the terminus of both O- and N-linked glycans (Varki, 2007). Below is a brief discussion on the potential effects that SSL11 may have on molecules that express sialylLacNAc.

i) Sia-recognising Ig-superfamily lectins (Siglecs)

Siglecs are a recently described class of animal proteins with binding specificity to sialated carbohydrates. A subset of siglecs have cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIMs - S/I/V/LxYxxI/V/L) or ITIM-like motifs. The siglecs exhibit differences in their carbohydrate binding specificity (figure 6.2). Immune processes associated with the activation of siglecs by ligation with Sia and subsequent phosphorylation of the ITIMs include inhibition of T cell receptor signaling and NK cell-mediated cytotoxicity (Ikehara et al., 2004; Nicoll et al., 2003). Nicoll and colleagues (2003) found that siglec function is blocked by the binding of endogenous ligands, masking their ability to regulate NK cells. Neuraminidase treatment of NK cells exposed the binding site of siglec-7, increasing binding of a glycoprobe. It is therefore a possibility that SSL11 may compete for binding of siglecs to their endogenous ligands, therefore exposing their Sia binding site and allowing their activation to inhibit cellular function.
The crystal structure of the N-terminal domain of mouse sialoadhesin (siglec-1) in complex with its ligand, sialyllactose, has been solved to 1.85Å (May et al., 1997). As expected, the majority of interactions between siglec-1 and sialyllactose are to Sia, which includes six hydrogen bonds and two hydrophobic interactions with nearby tryptophans. Gal contributes a single hydrogen bond to siglec-1 (figure 6.3). This is comparable to SSL11, where six hydrogen bonds are formed with Sia and two are formed to Gal. The similarities in binding observed in the crystal structures are further supported by similarities in binding kinetics. Siglec-1 binds to sialyllactose with a $K_D$ of $\sim 3.2 \times 10^{-6}$ M (Crocker et al., 1991; May et al., 1997), which is comparable to the dissociation constants of SSL11 to the related carbohydrate, sLe$^\alpha$ (25.4 ± 4.61$ \times 10^{-6}$ M for SSL11-GL10 and 0.85 ± 0.09$ \times 10^{-6}$ M for SSL11-US6610). Additionally, siglec-8 specifically bound to Neu5Aco2–3(6-O-sulfo)Galβ1–4[Fuc1–3]GlcNAc (6'-sulfo-sLe$^\alpha$) with a $K_D$ of 2.2-2.3$ \times 10^{-6}$ M (Bochner et al., 2005). This indicates that SSL11 may be able to compete with siglecs for binding to common either endogenous or exogenous ligands.
Figure 6.2 - Binding specificities of siglecs
The strength of siglec binding to the glycans is indicated as follows: ++ strong binding; + detectable binding; –
very weak or no detectable binding (Figure from Varki and Angata, 2006).

Figure 6.3 - The binding site of sialoadhesin
Sialyllactose (yellow sticks) in complex with mouse sialoadhesin (green ribbon and sticks). Hydrogen bonding
is represented as dashed lines with distances in Å. * indicates the Arg residue conserved in all siglecs. Image is
generated from pdb file 1QFO (May et al., 1997)
ii) Selectins

The crystal structures of P- and E-selectin in complex with sLe\(^\text{x}\) have been solved to 3.4 Å and 1.5 Å respectively (Somers et al., 2000) and show a high similarity in their binding modes (figure 6.4). Both P- and E-selectin chelate a single Ca\(^{2+}\) ion, which is bound to Glu and Asp residues on the protein surface. The interaction between P- and E-selectin to sLe\(^\text{x}\) is calcium-dependent, which is reflected by the strong interaction between the covalently-bound Ca\(^{2+}\) ion and Fuc. E-selectin exhibits additional hydrogen bonding to Sia and Gal of sLe\(^\text{x}\), compared to P-selectin, which may explain the 10-fold difference observed in K\(_D\) values (Poppe et al., 1997). PSGL-1 binds to P-selectin with a K\(_D\) of 0.32 ± 0.02x10\(^{-6}\) M (Mehta et al., 1998). The binding of the sLe\(^\text{x}\) on PSGL-1 to P-selectin is primarily electrostatic and requires that proximal tyrosines on PSGL-1 are sulphated (Goetz et al., 1997). Zhang and colleagues (2004) propose that the dissociation of sLe\(^\text{x}\) from selectins involves overcoming two energy barriers. The inner barrier determines the dissociation rate at higher flow rates, where the Ca\(^{2+}\) ion was shown to provide a high tensile strength to the pulling forces. In contrast, the outer barrier determines the dissociation rate at lower flow rates, where the Sia moiety of sLe\(^\text{x}\) was shown to have an important role.

Figure 6.4 - Binding of E- and P-selectin to sLe\(^\text{x}\)

The sLe\(^\text{x}\) (yellow sticks) binding sites of A) E-selectin and B) P-selectin (green ribbons), with the chelated Ca\(^{2+}\) ion (red sphere). The sLe\(^\text{x}\) molecule is oriented with the Fuc directly above the Ca\(^{2+}\) (left) and Sia on the right. Images were generated from the pdb files 1G1T and 1G1R (Somers et al., 2000).
The mode of P-selectin binding to sLe\(^x\) is contrary to that exhibited by SSL11, where Sia is the primary sugar and the fucose is not essential. Notably, SSL11 was able to inhibit neutrophil rolling on a P-selectin surface, although P-selectin binds with a higher affinity to PSGL-1 than SSL11. The inhibitory effect was probably due to the much larger number of SSL11 molecules than PSGL-1 receptors. Neutrophils present 26500 ± 4500 PSGL-1 molecules on their surface (Kappelmayer et al., 2001), which for the sample of 10\(^6\) cells used in the in vitro rolling assay, gives an upper value of 3.1x10\(^{10}\) PSGL-1 per sample. A 50% inhibition of neutrophil adherence was observed with 50 nM SSL11, which equates to 3x10\(^{13}\) molecules per sample, 1000-fold greater than the number of PSGL-1 molecules.

It is also likely that SSL11 is able to inhibit NK cell and T and B lymphocyte rolling. T-lymphocytes and NK cells express 38200 ± 26000 copies of PSGL-1 and B lymphocytes express 2600 ± 1500 copies on their surface (Kappelmayer et al., 2001). P-selectin is involved in the initial interaction of both T and B lymphocytes to endothelial cell layers (Yago et al., 1997; Yago et al., 1995). Additionally, activation of B lymphocytes resulted in an increase in sLe\(^x\) surface expression, which correlates with the observation that active, but not resting B cells are able to interact with P- and E-selectin (Postigo et al., 1994).

### iii) Gangliosides

Gangliosides are sialated glycosphingolipids (oligosaccharides coupled to ceramide, also known as sphingosine) and are ubiquitous on mammalian cells plasma membranes. They are reported to be involved in a broad range of cell processes including growth, adhesion, and signaling (Iwabuchi et al., 1998; Miljan and Bremer, 2002). For the purpose of this discussion, I will focus on the ganglioside GM3 (Neu5Acα2-3Galβ1-4Glcβ1-1Cer). GM3 is present on granulocytes and monocytes and is the major ganglioside constituent in lymphocytes (Kiguchi et al., 1990).

Type 3 “glycosynapses” are formed by the clustering of integrins with glycosphingolipids, with the molecular facilitator tetraspanin, in plasma membrane microdomains (Hakomori, 2004; Maecker et al., 1997). Glycosynapses control carbohydrate-dependent cellular processes, such as cell-cell interaction and cell signaling.
Several ligands and associated molecules have been identified for GM3, including the globoside Gg3 (GalNAcβ1-4Galβ1-4Glcβ1-1Cer), growth factor receptors, the T cell marker CD4, and the chemokine receptor CXCR4 (fusin). GM3 is involved in cell-cell interactions, as observed between mouse melanoma B16 and mouse lymphoma L5178. The B16 cells express high levels of GM3 and the L5718 cells express high levels of Gg3 (Kojima and Hakomori, 1989). This adhesive interaction can be inhibited by the addition of either GM3- or Gg3-liposomes, or antibodies against GM3 or Gg3. GM3 and Gg3 form a strong interaction with a $K_D$ of 0.4x10^{-6} M (Matsuura and Kobayashi, 2004). The adhesive effect of GM3 to Gg3 is synergistic with integrin binding to adhesive matrix proteins e.g. fibronectin and laminin (Kojima and Hakomori, 1991).

Addition of GM3 into the growth medium of epidermoid carcinoma cell lines KB and A431, hamster fibroblast line BHK, and mouse Swiss 3T3 cells, significantly inhibited growth (Bremer and Hakomori, 1982; Bremer et al., 1984; Bremer et al., 1986). The reduction in growth was the result of inhibition of ligand-induced phosphorylation of receptors for fibroblast growth factor, epidermal growth factor and platelet-derived growth factor.

GM3 is implicated in several functional pathways of T cells. Treatment of T lymphocytes with GM3 caused a significant increase in CD4 phosphorylation, an event associated with subsequent endocytosis and degradation of CD4 (Sorice et al., 2004). Additionally, the Syk-family kinase, Zap-70 is translocated from the cytoplasm to plasma membrane microdomains during T cell activation, where it associates tightly with GM3 (Garofalo et al., 2002). Phosphorylation of substrates by Zap-70 results in the amplification and diversification of T cell receptor signaling.

Because SSL11 is able to bind to sialyllactose (the oligosaccharide portion of GM3) there are numerous implications for functions of SSL11. Not only is there potential for SSL11 to disrupt cellular adhesion, growth and signaling, there is also potential for targeting of SSL11 to tissue types. For example, >75% of the SAg, SEB, accumulates in the kidneys of rabbits and monkeys as a result of interaction with the glycosphingolipid, digalactosylceramide (Chatterjee et al., 1995; Israel et al., 1961; Rapoport et al., 1967).
iv) Sia-dependent adhesion of *S. aureus*

*Staphylococcus aureus* is known to invade host cells to avoid recognition by leukocytes (Bayles *et al.*, 1998; Ogawa *et al.*, 1985). Several other bacteria specifically target and invade cells through Sia-dependent mechanisms. *Anaplasma phagocytophilum* is the causative agent of the potentially life-threatening disease granulocytic ehrlichiosis. In human, *A. phagocytophilum* binds to neutrophils in a sLe^x^-dependent manner (Yago *et al.*, 2003). In addition, the gram negative bacterium *Helicobacter pylori* binds to the disaccharide Neu5Aca2-3Gal on gastric cells via its surface protein, SabA (Aspholm *et al.*, 2006; Roche *et al.*, 2004).

Expression of sLe^x^ and modified sLe^x^ molecules was induced on endothelial cell surfaces in inflamed tissues (Renkonen *et al.*, 2002). The proportion of vessels expressing sLe^x^ to modified sLe^x^ (i.e. sulphation) has been proposed as a means of zip coding endothelium, as different organs exhibited different ratios (Renkonen *et al.*, 2002). Although SSL11 is a secreted protein, there is evidence of its presence on the surface of *S. aureus* (Laughton *et al.*, 2006). Therefore, it is possible that *S. aureus* uses SSL11 as an adhesin, allowing it to bind and interact with endothelium in specific organs, expressing compatible carbohydrate on their surface. This correlates with the increased virulence of *S. aureus* and its ability to adhere to surfaces, upon expression of SSL11 (Gan *et al.*, 2002; Laughton *et al.*, 2006).

6.5 Internalisation of SSL11

SSL11 was rapidly internalized into neutrophils by a mechanism that is dependent on its carbohydrate-binding site. Once internalized, it exhibited a cytoplasmic distribution with exclusion from the nucleus. Modulation of the proteome occurred within 1 h and the most notable effect was on actin. It is possible that the effect on actin was due to actin remodeling associated with vesicle formation, as seen by the localization of SSL11 in brightly-stained cytoplasmic vesicles (figure 5.6C). Alternatively, SSL11 may impair actin polymerization through its modification by ADP-ribose. Further investigation into the type of modification undergone by actin could be performed by determining the incorporation of radioactively-labeled phosphate, ADP-ribose or ATP-ribose.
6.6 Future directions

i) Identifying a receptor for SSL11 on *S. aureus*

Although SSL11 contains the consensus sequence for secretion and has been identified in *S. aureus* supernatant, Laughton and colleagues (2006) originally identified SSL11 as a surface-bound protein. There are three potential mechanisms for this. The first is a covalent linkage of SSL11 to the surface. SSL11 lacks the LPXTG motif that allows for sortase-dependent covalent linkage to the bacterial surface, which makes this option unlikely. Second, the surface of *S. aureus* was reported to be highly hydrophobic and negatively charged (Dickson and Koohmaraie, 1989), which may result in non-specific interaction of proteins with the bacterial surface. The calculated pI of SSL11 alleles is between 5.5 and 8.8, but it is likely there would be regions of both high positive and negative charge. Third, a surface-bound receptor exists for SSL11. This could be investigated by determining if SSL11 binds to *S. aureus* in a saturable manner and if different strains bind different amounts of SSL11.

ii) Determining the interaction of SSL11 with other SSLs

The *SaeRS* TCRS regulates the expression of SSL11, but appears not to be involved in the expression of other SSLs. However, SSLs 1-4 are expressed during mid-log phase, with increased expression during the stationary phase (Fitzgerald *et al.*, 2003). Additionally, SSLs 2, 3 and 5 are upregulated by *S. aureus* over a period of 180 min after phagocytosis by neutrophils (Voyich *et al.*, 2005). The shared temporal expression by SSLs begs the question – do they interact with each other? The crystal structures of SSLs 5, 7 and 11 all indicate formation of a homodimer (Al-Shangiti *et al.*, 2004; Arcus *et al.*, 2002), but can they also form heterodimers? SSL11 and SSL5 form the most similar homodimers, both with six hydrogen bonds across the β7- strands, between the peptide chains. Because the side chains aren’t directly involved in dimerisation, it is possible that an SSL11-SSL5 heterodimer could form. The formation of SSL heterodimers would allow for a broad range of function, in terms of cross-linking important molecules or cells. For instance, cellular staining was observed within 30 s on ice for SSL11 compared to SSL7, which required longer incubations of higher concentrations to achieve reasonable staining (Al-Shangiti *et al.*, 2004). An SSL11-SSL7 heterodimer would allow SSL7 to interact at the cell surface at a much faster rate, possibly increasing its potency. This could be tested by simple ELISA assays to determine if labeled SSLs interact with non-labeled SSLs coated to the plate.
iii) Designing inhibitory drugs against the SSLs

SSLs are likely to be important virulence molecules that specifically target various immunologically-important molecules. A common binding mode for a sub-family of SSLs has been identified, which could potentially be used as a target for drug design. Preliminary testing has confirmed that SSLs 2-6 do indeed share carbohydrate specificity with SSL11 so that a simple inhibitor of seven separate SSL virulence factors is entirely feasible. At least for SSL11, the primary mode of interaction with cell surfaces is through this single binding site, as the knockout mutant SSL11-T168P failed to stain human neutrophils and failed to inhibit neutrophil adhesion to a P-selectin coated surface. Impaired binding of SSL11 results in its inability to internalize and affect intracellular pathways in addition to its effects on leukocyte rolling. Several Sia mimetics have been produced and marketed for influenza treatment, such as Relenza (zanamivir) and Tamiflu (oseltamivir), that target the viral neuraminidase (Lynch and Walsh, 2007). A more complex small molecule mimetic, containing an α1-4 bond to galactose-like sugar would provide ample and specific inhibition of SSL binding. For example, replacing the hydroxymethyl group of Gal with an aminal group could form two hydrogen bonds with the fully conserved Glu at position 170 in SSL11-US6610 (figure 6.5). Screening would be simple to perform by competition of SSL11 binding to sLe\(^\times\) by surface plasmon resonance.
iv) Defining the role of SSL11 in phagocytosis

Two receptors involved in phagocytosis (FcαRI and Mac-1) have been identified as ligands for SSL11. Notably, the association of Mac-1 with FcαRI confers the ability for FcαRI to bind to secretory IgA (sIgA), which is the primary antibody at mucosal surfaces (Van Spriel et al., 2002).

Opsonisation of pathogens enhances their susceptibility to phagocytosis. The two main opsonins are the complement factor C3bi and immunoglobulins, including IgA. Phagocytosis of *S. aureus* opsonised with either IgA or sIgA was significantly greater than non-opsonised bacteria (Gorter et al., 1987). In addition, phagocytosis was almost twice as efficient when *S. aureus* was opsonised with sIgA, compared to IgA. However, both IgA- and sIgA-opsonised bacteria were able to induce comparable amounts of reactive oxygen species (ROS) release (Gorter et al., 1987). It has since been found that induction of ROS by sIgA requires association of FcαRI with Mac-1 (Van Spriel et al., 2002).
Experiments revealed that SSL11 inhibited IgA binding to FcαRI, which would potentially impair IgA-mediated opsonophagocytosis. In addition, the literature supports an increased virulence of strains expressing the SaeRS locus (which primarily upregulates SSL11) that may be a result of decreased ability of neutrophils to phagocytose them (section 1.5.4 v). Therefore, further investigation into the effects of SSL11 on phagocytosis would be important in determining further function.

v) Determining the effect of SSL11 on actin
Analysis by 2-D gels showed that upon internalization, SSL11 mediated changes in actin. The type of modification that actin underwent is unknown, as is whether the effect was mediated through direct interaction, or through actin-associated proteins. Because of the importance of the actin cytoskeleton on cellular function, further characterization of the effects of SSL11 on actin would be a key step towards determining the intracellular function of SSL11. For instance, the inhibition of actin polymerization, or the depolymerisation of actin filaments would have major consequences for the ability of immune cells to respond to bacterial infection. In addition, the activation of actin remodeling may aid in the non-phagocytotic internalization of S. aureus. The type of actin-modification could be determined by the incorporation of radioactively-labeled ATP-ribose, ADP-ribose, or inorganic phosphate and exposure to film.

6.7 Conclusions
SSL11 is an important virulence factor produced by S. aureus. Its expression is directly correlated with virulence and the data presented within this thesis indicate it has an important role in leukocyte recruitment and modulation of the neutrophil proteome. In addition, a common binding mechanism was identified for a subset of SSLs. This shared binding mode has implications in the targeting of SSLs to leukocytes where their true functions may be revealed.
### 7.1 Oligonucleotides

<table>
<thead>
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<th>Primer name</th>
<th>Primer sequence (5′-3′)</th>
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<td>SSL11-N315-Fw</td>
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<tr>
<td>SSL11-N315-Rv</td>
<td><strong>CAGGAAAACAGCTATGAC</strong>GAATTCCGAATAATTTTATAAATTCACTTT</td>
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<td>BirA-Rv</td>
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<tr>
<td>T168P-Fw</td>
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</tr>
<tr>
<td>T168P-Rv</td>
<td><strong>CTTTTTATTTATTTTTGAAT</strong></td>
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</tbody>
</table>

Primers are labeled Fw (Forward) or Rv (Reverse) with M13 tags in *italic*, restriction sites in *bold* and complementary sequences *underlined*. Annealing temperatures were designed to be around 50 °C.
7.2 Plasmids

pBluescript and pBC are from Stratagene.
pGEX3C was modified from pGEX-2T (Amersham) by Dr. Thomas Proft.
pET32a3C was modified from pET32a (Novagen) by Dr. Thomas Proft.
CMV P = cytomegalovirus promoter
EC1 EC2 = extracellular domains 1 and 2 of FcαRI
IRES = internal ribosome entry site
Neo = neomycin resistance
polyA = polyadenylation site
AMP = ampicillin resistance

pBAR266 was modified from pIRESneo by Dr. Bruce Wines
7.3 *S. aureus* strains

Clinical isolates from Greenlane Hospital, New Zealand

NU4318  NU4410  NU4247  NU4362  NU4424  NU4427  
NU4483  NU4487  NU9093  NU9138  NU9278  NU9279  
NU9281  NU9282  NU9304  NU9324  NU9325  NU9341  
NU9365  NU9380  NU9419  NU9425  NU9444  NU9617  
NU9626  NU9637  NU9638  GL10

Clinical isolates from Auckland Hospital, New Zealand


Clinical isolate from unknown source, New Zealand

SAPRI

Clinical isolates from various German hospitals

47547103  13773  B970  F14

Clinical isolates from U.S.A.

SEG    SEH    SEI 445    SEI F127    ATCC33593

7.4 Serum samples from *S. aureus*-infected patients

Samples were from patients in Auckland, New Zealand.

HMR0800 - *S. aureus* bacteraemia arising from peripheral IV line sepsis

HGV3492 - *S. aureus* bacteraemia

MND6089 - *S. aureus* endocarditis

DEC6517 - *S. aureus* scalp wound infection
7.5 Seroconversion

SSL5 seroconversion dilution series

SSL11-GL10 seroconversion dilution series

SSL11-US6610 seroconversion dilution series
7.6 MALDI-MS Results

Sample #2

Match to: TRFL_HUMAN Score: 174 Expect: 6e-014
Lactotransferrin precursor (EC 3.4.21.-) (Lactoferrin) (Talalactoferrin alfa) [Contains: Kaliocin-1]
Nominal mass (Mr): 78132; Calculated pI value: 8.50
NCBI BLAST search of TRFL_HUMAN against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Homo sapiens
Variable modifications: Carbamidomethyl (C), Oxidation (M), Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KK unless next residue is P
Number of mass values searched: 200
Number of mass values matched: 65
Sequence Coverage: 63%
Matched peptides shown in Bold Red

1 MKLVTFYLLFL GAGGCLILAG RRPSQVCAN SQPEATKCFQ WQRNRKVRG
51 PPVSCIKROS PIQCTQAIIE HRADATVLD GFIYEAGLAP YKLRFVAAEV
101 YGTERQPRTY YYAVALVKRG GSQQLNAGQ IKSCTHTGRRR TAGKTVPIGT
151 LRPLNHWGFP PEPEEAARAV FFSAVCVFAP DKGQPFNLCD LCA27GENKC
201 AFQGSEVYFG YSAGAFCLRLD IAGDVAFIRE STVFEELSDAE AERDEYELLC
251 PDNTKPVDFK FDGCHLARVP SRAVARVSN SKEDAIWLLL ROQAEKFEGKD
301 KSPKQMFLGS PSQGKDLLFF KSAIGFSRVDP PRIDSGLYLG SGYFATIQNL
351 RKSEEEVAR LARVRWCAYQ EQELRKNQW SGLSEGSTC SSSASTEDCI
401 ALVLRGEADA MSLOQGYYVT AGKCVLFFVL AENYKRSQSS DPDEFKCVDRP
451 VEGYLAVAVY RRSQTSIZWN SVKQKSCHT AVTRTAGWNI RMGLNPWQTG
501 SCKSFDYSFQ SCAPGDSPRS NLACALC1GDE QGENKCPVFN3 NERVYGGTTSA
551 FRCLEAQAGD VAFKVDTVL QNTGNNNNEA WAKDLKLDFF ALLCLDGKRK
601 PVTSEARSCHL AMAPNHAVVS RDQVERLQ VLLHQQARFQ RNGSDEPPKF
651 CLFQSETKYL LFNDNTECLA RLHGKTTYEK YLGPQIVVAGI TNLKRCSTSP
701 LLEACEFLRK

MC2

4700 Reflector Spec #1 MC[BP = 1007.5, 54064]

- 144 -
Sample #3

Match to: TRFL_HUMAN  Score: 145  Expect: 4.8e-011
Lactotransferrin precursor (EC 3.4.21.--) (Lactoferrin) (Talalactoferrin alfa) [Contains: Kaliocin-1]
Nominal mass (M): 78132; Calculated pI value: 8.50
NCBI BLAST search of TRFL_HUMAN against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Homo sapiens
Variable modifications: Carbamidomethyl (C), Oxidation (M), Propionamide (C)
Cleave by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 200
Number of mass values matched: 59
Sequence Coverage: 59%
Matched peptides shown in **Bold Red**

1 MKLVFLVLLF LGALGLCLAG RRRSVQWCAV SQPEATK CFQ WQR NMRK VRG
51 FPVSCIKRDS PIQCIQAIIE NRADAVTLDG GFIYEAFLP YKLRFVAEEV
101 YGTERQPRTH YYAVAVVKKG GSFOFNLLOQ LKSCHTLGRK TAGVNPIGT
151 LRPLLWINWG PEPIDEAAVAR FFSASCVFGA DKGQFPNLKR LCAQTGENKC
201 AFSQGPPFPS YSGAPKCLSD GAGDVAFIRE STYFEDLSDE AERDEYELLC
251 PDNTRKPVDR FRDCHLARVP SHAVARSVN QKEDA1WLLL RQAGEKFGKD
301 KSPKFGQFLSF PQQKDLHFK DSAIGFSRVV PRIDSGLYLH SGYFTAIYNL
351 RKSEEVEAAA RARUVWCGAV EQELRKCQKW SGLRGLSSVTCS SASTTEDCI
401 ALVKLHDEA MSLQDGIFVT AGKCLGVPVL AENYKSQGFS DPVFNCDVRF
451 VEQVLAVAVV RRSDETLWN SVKGRKSCHT AVDRTAGYNT PMLLFQNTFG
501 SCKFSEYSGQ SCAGPSDPRS NLCAICIGDE QGENKCVPSN RERYGYTGA
551 FRCLENAEAD VAFVYDKVTVL QNTQNENNEA WAKDLKLADF ALLCLDGKRK
601 FTVSEARSCH AMPNHAUVS RMDKVERLQK VLLAQKAPFK RMSGDCPDKF
651 CLFQSEKXNL LFNDNTECLA ALHGKTYYEK YLGFPQVAGI TNLKCSSTSP
701 LLEACEFLKR

MC3

4700 Reflector Spec #1 MC[BP = 1097.5, 39778]
Sample #4

Match to: ITAM_HUMAN  Score: 152  Expect: 9.5e-012
Integrin alpha-M precursor (Cell surface glycoprotein MAC-1 alpha subunit) (CR-3 alpha chain) (Leuk)
Nominal mass (M): 127099; Calculated pI value: 6.88
NCBI BLAST search of ITAM_HUMAN against nr

Unformatted sequence string for pasting into other applications

Variable modifications: Carbamidomethyl (C),Oxidation (M),Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 200
Number of mass values matched: 59
Sequence Coverage: 48%
Matched peptides shown in **Bold Red**

```
1 MALRVLVLLTALTITCGLFNLD TENAMTFQEN ARFGGQGSVQV LQSGRNVVGA
51 PQEIVAANQR GSLYQCDYST GSCFIRIQLV PVEAVNMSLG LSLAATSSPP
101 QLACGPTVRH QTCESENTYVK GLCFLFGSNL RQQPKQPEEA LRGCFQEDSD
151 IAPFLDGSIG IPHQDRFMK EVFSTVEMLQ KSKRLFLSML QYSEFRRINF
201 TPFPCFQVPH PRSLVPRIPTP LGLRTHATG IRKVQPELFW ITGAKRNAP
251 KLVITGIDK KFDPDLYTED VEPAEDEREG IRYIVGVQDRA FRSKSRQEL
301 NTLASKPFRD HLVQVNNFEEA LKTIQQLKRE KIFAEGETQ GSSSSEHEHM
351 SQGSFSAATQ SNQPLLSTTVG SYDWAAGVFL YTSKEKSTFI NMTVDSDMN
401 DAVLGYAAAIL ILMRVQSVLV LGAFPYQH LIVAMFRQNTG WHNEWANVKG
451 TQGAYVPGAS LCSRVDVDSNG STELVLIGAP KHEYQTRBGQ VSCVPILPRG
501 ARWQDCAVLY GEQQPQWGRF GAALTVLGDV NDKLITDVAI GAGEEDNRRG
551 AVYLFHRSTSG SISPSSHRQR JAGSKLSPRL QYFQQLSLSG QDLMGDLVD
601 LTVAGQGQVLK LLRSQPVLVRK TALEMPFRP VARNVFCNQ VQVRSKEAGE
651 VRVCNLHVQKS TRPLARQEQI QSVVTYDLAL DSQFRHSRAV FNETRAYSTAR
701 TQGQLYQTPC CTPLIQQFLNP LSNPISLQLT GLSAPQNLAP
751 VLADEAQFLP TALFFFEKNC GNDNPCQDDL SITPSFMSLD CLVYGVRPRFL
801 NVTVRTVRDG ED SlyR TQVTF FFPPLDLSYRK VSTLQQRQSQ RSWRLACEA
851 SSTVEVSGARK STSCSINHIP PESENVEFPN ITFDVOSKAS LGNLRLKAN
901 VSEVENKASQ PTNTEQGQLP VYKAYVNTT SHVSTVYKLN PTASENTSAR
951 MQOYOQVGLG QQRSLEISLFL VLFVPRLNQG VITRDFPQVTF SENLSSTCHT
1001 KERLPSHDG LAFLEKAPPV NCSIAVQGQ QCDBGFGIQ FEENATLKEN
1051 LSLFDWYIKTS NHNLIVSTTA ELFNSVFTV LFPPGQAFRV SQETKVEFFP
1101 EVNPNFLPFLV GSSEGLLLLL ALITAALYKL GFFKQYKDM MSEGPPGAE
1151 PQ
```

MC4

4700 Reflector Spec #1 MC (BP = 1324.8, 20803]

- 146 -
Sample #6

Match to: ITA2B_HUMAN Score: 146 Expect: 3.8e-011
Integrin alpha-IIb precursor (Platelet membrane glycoprotein IIb) (GPIIb) (GPIIb) (CD41 antigen)
Nominal mass (Mr): 113320; Calculated pI value: 5.21
NCBI BLAST search of ITA2B_HUMAN against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Homo sapiens
Variable modifications: Carbamidomethyl (C), Oxidation (M), Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 121
Number of mass values matched: 41
Sequence Coverage: 44%
Matched peptides shown in Bold Red
1 MARALCPLQA LWLEWLLL LGPCAAPAW ALNLDPVQLT FYAPFKGSQF
101 GFSLDHKDS IGRVAIVVGA PRTLGSQEE TGGVFLCPWR AEGQCQPSLL
151 FFDRDTRNV GQQLQLQFKA RQQLGASSVWS WSDTIVAVAP WQRMMNLKRT
201 EEAKTPFGS CFPALQVESGR RAEXSCPGR NLSPRTVEPEND FSFMDKRYCEA
251 GFSVYTGQAG EULVGAQAPE YPLGLAAQAP VAQIFSAYPR QILLNHVSSQ
301 SLGFDSNNPE YFDQYKQVSV AVGQFDGGDL TTEYYVQAGT WSWTQAVGSI
351 LQYYRLHR QAERQMSAYF GHSVAQTDVN GDGRHDILVG APLYMERSAD
401 RKLAEVFKV LVLPXQAPGPA LGAPSLLLTG TQLYGRFQSA IAPQGDLDRD
451 GYNDIAAVP YGGFGSRGQV LFLQGQSEGAR ASRPSQVGLDS PFFPQAFGSF
501 SSLGIVDIDD NVGPGDLVIGA YGANGQVYRR ACPYVQASVQ LBLQGSLNPA
551 VQSCVLQPTK TPVSCFNIQM CVGATGHNP QKLSNAEQLQ DORQPRQGR
601 RVLLKSSQGA GTLNLDLLLL KHSFICTTM AFLR DEADFR DKLSQVLILSL
651 NVLSHPTEG MAPAVLHGD TVQEQTRIV LCQGDDVCV PQLQILASVT
701 GSFPLNOQKN EQLQQDQADN ESEGAYEAL AVHLQPGAKY MPAALNVEGF
751 RKLQGKSNQ NPNSKTVLDD VPVRAEQAE QLEGNFQPSL VVAAEGERE
801 QNSLDSQGPK VEHTYELHHN GPTVGNLH LHSLQGQSQP SDDLYLIDIQ
851 PQGGLQCFQF PFPVLPKLVWD GLPISPSFSPI HPAHRKRDQ QIQLPFEQQP
901 SRQDPVFLVS CDSAPCTIVQ CDLQBMAR QQ RAMTVLAFL WILPSLYQFL
951 EQPVJLQAMFW PNVSSLFVPV PFLSLPRGEA QVWTQQLDAL EERAAPFWWV
1001 LVVGLGQLLL LTLTVLAMWR VGFFKRNPP LEEDOEGR

4700 Reflector Spec #1 MC[BP = 1502.8, 26411]
**Sample #8**

Match to: TRFL_HUMAN Score: 162 Expect: 9.5e-013
Lactotransferrin precursor (EC 3.4.21.-) (Lactoferrin) (Talalactoferrin alfa) [Contains: Kaliocin-1]
Nominal mass (M): 78132; Calculated pI value: 8.50
NCBI BLAST search of TRFL_HUMAN against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Homo sapiens
Variable modifications: Carbamidomethyl (C), Oxidation (M), Propionamide (C)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: 200
Number of mass values matched: 58
Sequence Coverage: 64%
Matched peptides shown in **Bold Red**

1. MKLVFLVLLF LGAALCLAG RRR SVQWCAV SQPEATKCFQ WQR MKLVFLVLLF LGAALCLAG RRR SVQWCAV SQPEATKCFQ WQR

**4700 Reflector Spec #1 MC [BP = 1615.9, 24867]**

MC8

![Image of mass spectrum](image-url)
## 7.7 Glycan array v2.1

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<th>[SSL11-US6610] (RFU) 200 μg/mL</th>
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<th>2 μg/mL</th>
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**Spacer definitions**

| Sp0 | -CH₂CH₂NH₂                   |
| Sp8 | -CH₂CH₃CH₂NH₂                 |
| Sp9 | -CH₂CH₂CH₂CH₂CH₂NH₂           |
| Sp10| -NHCOCH₂NH                    |
| Sp11| -OCH₂C₆H₄-p-NHCOCH₂NH         |
| mMDPLys| -Mur-L-Ala-D-iGlnb-(CH₂)₂NH₂ |
| T   | -Threonine                    |
| N   | -Asparagine                   |
7.8 QStar Results

Spot #1 – Most likely result

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<th>Mr(calc)</th>
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<th>Peptide</th>
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Match to: gi|62897409 Score: 367
beta actin variant [Homo sapiens]

Nominal mass (M_r): 42038; Calculated pI value: 5.29

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Acetyl (Protein N-term), Deamidated (NQ), Gln->pyro-Glu (N-term Q), Glu->pyro-Glu (N-term E), Oxidation (M)
Semi-specific cleavage, (peptide can be non-specific at one terminus only)
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 25%

Matched peptides shown in Bold Red

1 MDDIAALVV DNGSGMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMQK
51 DSYGDEAQS KRGILTLYKYP IEHGIVTNWD DMEKIPvWHTF YNELRVAPEE
101 HPVLITEAPL NPNAKREKMT QIMFETFNTP AMYVAIQAVL SLYASGRTTG
151 IVMDSGDGGVT HTVIPYEYGA LPHAILRLDL AGRDLTDYLM KILTERGYSF
201 TTTAEREIVR DIKEKLICYVA LDFEQEMATA ASSSLEKSY ELPDQVITI
251 GNERFRCPEA LFQPSFLGME SGGIHETTNF SIMKCDVDIR KDLYANTVLS
301 GGTMTYPGIA DRQKEITAL APSTMKIKII APPERKYSWV IGGSVLASLS
351 TFQMWN1SKQ EYDESFPSIV HRKCF

- 156 -
## Spot #2 – Most likely result

### Peptides

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**Match to:** gi|40226101  **Score:** 316  
**ACTG1 protein** [Homo sapiens]

Nominal mass ($M_r$): **29678**; Calculated pI value: **5.50**

**Taxonomy:** [Homo sapiens](https://www.ncbi.nlm.nih.gov/)  
**Fixed modifications:** Carbamidomethyl (C)  
**Variable modifications:** Acetyl (Protein N-term), Deamidated (NQ), Gln->pyro-Glu (N-term Q), Glu->pyro-Glu (N-term E), Oxidation (M)  
**Semi-specific cleavage:** cuts C-terminal side of KR unless next residue is F  
**Sequence Coverage:** 27%

**Matched peptides shown in Bold Red**

1. KANREKMTQIFMETNTPAMVYAIQAVLSLYASGRTTGIVMDSDGDVTHT  
2. VPI YEYALPAILRLDDLARGDLTDYLMKILTERTYSGFTTAEIEVRDI  
3. KEKLCYVALDFEQEMATAASSSLEKSYELPDQVITIGNERFRCPFEALF  
4. QPSFLGMESCIGHETFNSMKMCVDIRKDLVNTVLSGGTTMYPGIADK  
5. MQREITALAPSTMKIIAPPERVERKYSWIGGSILASSTFQQWMISKQEY  
6. DESGPSIVHRKCF
Spot #3 – Most likely result

Peptides

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Match to: gi|40226101 Score: 317
ACTG1 protein [Homo sapiens]

Nominal mass (Mr) : 29678; Calculated pI value: 5.50

Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Acetyl (Protein N-term), Deamidated (NQ), Gln->pyro-Glu (N-term Q), Glu->pyro-Glu (N-term E), Oxidation (M), Phospho (ST), Phospho (Y)
Semi-specific cleavage, (peptide can be non-specific at one terminus only)
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 24%

Matched peptides shown in **Bold Red**

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101 KEKLCYVALD FEQPENYTAAS SSSLEKSYEL PDQVITIGN ERFBCPEALF
151 QPSFIQEMSC GINETTFNSI MKCDVDIRKD LYANTVLSGG TTYMPIGADR
201 MQEITALAP STMKIIAIP PERKYSVWIG GSILASLSTF QQMWISKQEY
251 DESGPSIVHR KCF
### Peptides

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Match to: gi|62897409 Score: 465
beta actin variant [Homo sapiens]
Nominal mass (M_r): 42038; Calculated pI value: 5.29
Taxonomy: Homo sapiens

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Acetyl (Protein N-term), Deamidated (NQ), Gln->pyro-Glu (N-term Q), Glu->pyro-Glu (N-term E), Oxidation (M)

Semi-specific cleavage, (peptide can be non-specific at one terminus only)
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 31%

Matched peptides shown in **Bold Red**

1. **MDDDIAALVVDNGSGMCKAG FAGDDAPRAV FPSIVGRPRH QGVMVGMQK**
2. **DSYVGDEAQS KRGIIITLKYIP IEHGIYVYNW DMEKIWWHTFY YNELRVAPEE**
3. **HPVLLTEAPLN NPRAMREKMT QIMFETFTNTP ANYVAIQAVVL SYASGRTTG**
4. **IVMDSSQDCGHT HTVIYEGYIA LHAILRLDL AGRLDLTDYLM KILTERCYSF**
5. **TTAEREVIR DIKEKLCYVA LDFQEFQMAT AASSSLEKSY ELPDQGQVITI**
6. **GNERPCRPEA LFQPSFLGME SCCHETTFN SIMKCDVDIR KDLYANTVLS**
7. **GTTMTYPGIA DRMQKEITAL APSTMKIKII APPERKYSVW IGGSVLASLS**
8. **TFQQMWSKQ EYDESFPSIV HRKCF**
Chapter 8 - Bibliography


Rosenbach (1884). Mikroörganismen bei den Wundinfektionskrankheiten des Menschen.


Wines, B.D., Willoughby, N., Fraser, J.D., and Hogarth, P.M. (2006). A competitive mechanism for staphylococcal toxin SSL7 inhibiting the leukocyte IgA receptor, Fc alphaRI, is revealed by SSL7 binding at the C alpha2/C alpha3 interface of IgA. J Biol Chem 281, 1389-1393.


