Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage.
http://researchspace.auckland.ac.nz/feedback

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.
The interaction and influences of Staphylococcal Superantigen-Like Protein 11 with myeloid cells

Richard Peter Sequeira

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science, The University of Auckland, 2013.
Abstract

*Staphylococcus aureus* can cause severe diseases with symptoms ranging from superficial skin infections to infective endocarditis and toxic shock syndrome. A family of 14 virulence factors known as Staphylococcal Superantigen-Like proteins (SSLs) have been identified that have affinity for molecules of the innate immune system and aid with immune evasion. A clade within the SSL family has been identified to bind carbohydrates. SSL11, a member of this clade, is able to bind sialyllactosamine (sLacNAc) with high affinity. Sialylation is an important glycosylation of immune receptors as the carbohydrate is directly involved in immune recognition and signalling. It is therefore hypothesised that SSL11 helps the bacterium evade immune surveillance by interfering with the immune recognition of sialylated receptors and their subsequent responses. This thesis sought to further investigate the influence SSL11 has on immune function.

SSL11 binding to Sialyl Lewis X (sLe^x^) exhibited no differences in affinity when compared to SSL11 binding sLacNAc. This lack of specificity for additional sugars explains why SSL11 does not compete or saturate receptors on the surface of cells, as it can promiscuously bind receptors exposing α2-3 linked sialylated galactose. SSL11 was observed to dimerise in solution as well as on the cell surface. The dimer exhibited prolonged binding to sLe^x^ with incomplete dissociation from the sialylated carbohydrate. The SSL11 dimer caused the aggregation of myeloid cells by cross-linking receptors on adjacent cell surfaces.

While SSL11 exhibited no effect on complement activation, apoptosis, platelet aggregation or phagocytosis, it did halt the migration of neutrophils to opsonised bacteria. This is not due to a loss in sensing the chemotactic anaphylatoxin C5a, but more likely due to the activity of SSL11 at the uropod which appears to tether the cells down at this location. This may relate to observed changes in actin in myeloid cells that have internalised SSL11. It is currently unknown if SSL11 perturbs receptor functions and subsequent cellular responses at the uropod or if it directly prevents the detachment of the neutrophil.

SSL11 internalisation into neutrophils and macrophages is via clathrin-dependent receptor-endocytosis. Following endocytosis, SSL11 travels in endosomes along the microtubule network to an unknown location. The most likely targets are the Golgi or lysosomal organelles. Internalisation of SSL11 appears to be associated with phenotypic cell changes.
and is completely absent in THP-1 monocytes. It is hypothesised that SSL11 is binding Mac-1 and using its activity to shuttle SSL11 into the cell.

The observed influences SSL11 has on neutrophil migration, in conjunction with aggregation, indicate that it could interfere with several steps in myeloid recruitment. This would be important in aiding the survival of the bacterium which is why SSL11 has been conserved in all observed isolates.
Acknowledgements

I would like to take the time to thank everyone that contributed to the project throughout my PhD. First, I wish to thank Professor John Fraser for giving me the opportunity to work on this project, and for his continued support and guidance. I appreciate the experience and am grateful to have been a part of the team. Thank you to my co-supervisor, A/Prof. Thomas Proft for supporting me and providing valuable advice.

Thank you to Dr Ries Langley and Dr Matthew Chung who had worked on SSL11 previously and created the foundation for my work.

To the Fraser lab, a big thank you for all your assistance, guidance, expertise and humour. I would like to thank Dr Deepa Patel for being a friend and mentor. I have learned so much from you and cannot stress how much I have valued your contribution. I would especially like to thank Dr Ries Langley, Dr Fiona Radcliff and Ms Fiona Clow for their expert mentorship on scientific writing. I would also like to thank my fellow PhD students: Ms Weilin Hou, Dr Stefan Hermans and Dr Natalie Lorenz, as well as past and present members of the lab, for their support and assistance throughout my project.

I would like to thank Ms Vicki Scott, Dr Natalie Lorenz and Ms Weilin Hou for always being available to take blood every week, without which, this project could not have continued.

A special thank you to Ms Jacqui Ross and Ms Hilary Holloway for your expert advice. It has been a joy and privilege to learn microscopy from you.

I would like to thank Professors Barbara Bröker and Uwe Völker for providing me the opportunity to come and work in your labs in Greifswald. To Dr Frank Schmidt, Dr Falko Hochgräfe, Dr Silva Holtfreter, Dr Dorothee Grumann and all members of the Bröker lab, I am grateful for all your support and appreciate the effort you put in to make me feel welcome.

Lastly, to my family, friends and anyone who has supported me, thank you.
# Table of Contents

Abstract ................................................................................................................................. ii
Acknowledgements ........................................................................................................ iv
List of Figures ................................................................................................................ xii
List of Tables .................................................................................................................... xiv
Abbreviations .................................................................................................................. xv

Chapter 1. Introduction ...................................................................................................... 1
  1.1. Overview .................................................................................................................... 1
  1.2. *Staphylococcus aureus* .......................................................................................... 2
    1.2.1. The organism ..................................................................................................... 2
    1.2.2. Pathogenesis of *S. aureus* ............................................................................. 2
    1.2.3. Antibiotic resistance ....................................................................................... 3
  1.3. The host immune response to *S. aureus* .............................................................. 5
    1.3.1. Attraction of neutrophils to the site of infection .............................................. 5
      1.3.1.1. Proinflammatory cytokines and chemokines ............................................ 5
      1.3.1.2. Complement ............................................................................................. 6
    1.3.2. Process of neutrophil migration ....................................................................... 8
      1.3.2.1. Molecules involved in neutrophil adhesion ............................................. 9
      1.3.2.2. Leukocyte diapedesis ............................................................................... 10
      1.3.2.3. Lymphocyte polarisation ......................................................................... 10
    1.3.3. Neutrophil killing of bacteria .......................................................................... 11
      1.3.3.1. Phagocytosis and intracellular bactericidal processes ............................. 11
      1.3.3.2. Extracellular killing mechanisms ............................................................ 11
    1.3.4. Importance of sialic acid in the immune system ............................................ 12
  1.4. Immune evasion by *S. aureus* ............................................................................... 14
    1.4.1. Immune evasive virulence factors ................................................................... 14
      1.4.1.1. Virulence factors that inhibit the chemotaxis of neutrophils ................. 15
      1.4.1.2. Virulence factors that inhibit opsonisation and phagocytosis ............. 16
      1.4.1.3. Virulence factors that prevent damage .................................................. 18
      1.4.1.4. Virulence factors that destroy immune cells ....................................... 19
      1.4.1.5. Virulence factors that modulate the adaptive immune system ............ 19
    1.4.2. Superantigens ................................................................................................... 20
      1.4.2.1. Structure of the superantigens ............................................................... 20
1.4.2.2. Function of the superantigens ................................................................. 21

1.4.3. Staphylococcal Superantigen-Like Proteins ............................................. 22
   1.4.3.1. Genomic organisation and regulation of the ssl family ..................... 24
   1.4.3.2. SSL immune-modulating cluster ....................................................... 25
   1.4.3.3. SSLs in the immune evasion cluster .................................................. 26
   1.4.3.4. Carbohydrate binding SSLs .............................................................. 26

1.5. Aims .............................................................................................................. 31

Chapter 2. Materials and Methods .................................................................... 32

2.1. Materials ...................................................................................................... 32
   2.1.1. Bacterial Isolates .................................................................................. 32
      2.1.1.1. Plasmids ......................................................................................... 32
      2.1.1.2. Bacterial strains ............................................................................. 32
   2.1.2. Protein Expression and Functional Analysis ......................................... 33
      2.1.2.1. Antibodies ..................................................................................... 33
      2.1.2.2. Common Buffers and Solutions ....................................................... 34
      2.1.2.3. Media .............................................................................................. 35
   2.1.3. Cell culture ............................................................................................. 36
      2.1.3.1. Media .............................................................................................. 36
      2.1.3.2. Cell lines ........................................................................................ 36
   2.2. Methods ..................................................................................................... 36
   2.2.1. Protein production ............................................................................... 36
      2.2.1.1. Inducing protein expression for purification in E. coli ....................... 36
      2.2.1.2. Nickel Affinity chromatography ..................................................... 37
      2.2.1.3. Anion exchange chromatography .................................................... 37
      2.2.1.4. Size exclusion chromatography ...................................................... 37
      2.2.1.5. Production of monoclonal anti-SSL11 ............................................ 38
   2.2.2. Protein Analysis ..................................................................................... 38
      2.2.2.1. Making acrylamide gels for SDS polyacrylamide gel electrophoresis (SDS-PAGE) ............................................................... 38
      2.2.2.2. Protein separation by SDS-PAGE ................................................... 38
      2.2.2.3. Staining of SDS-PAGE separated proteins with Coomassie blue stain .................. 39
      2.2.2.4. Staining of SDS-PAGE separated proteins with silver nitrate ......... 39
      2.2.2.5. Identification of SDS-PAGE separated proteins by Mass Spectrometry .......... 39
   2.2.3. Protein Modifications ........................................................................... 40
2.2.3.1. Coupling SSL11 to cyanogen bromide (CnBr) activated sepharose .................40
2.2.3.2. Fluorescein labelling of proteins.................................................................40
2.2.3.3. Biotinylation of SSL11 ................................................................................40
2.2.3.4. Attachment of a transferable linker to SSL11..............................................41
2.2.3.5. Alexa Fluor labelling of SSL11 .....................................................................41
2.2.4. Cell Culture ....................................................................................................41
   2.2.4.1. Isolation of plasma, mononuclear cells and granulocytes .......................41
   2.2.4.2. Isolation of platelet rich or poor plasma ..................................................42
   2.2.4.3. Isolation of serum ....................................................................................42
   2.2.4.4. THP-1 cell culture ....................................................................................42
   2.2.4.5. Differentiation of primary human monocytes to macrophages ..................43
   2.2.4.6. Bacterial growth .......................................................................................43
   2.2.4.7. Heat killing of S. aureus ............................................................................43
   2.2.4.8. Activation of serum complement by S. aureus ...........................................43
2.2.5. Analysis of Protein Interactions ......................................................................44
   2.2.5.1. Detergent lysis of purified cells .................................................................44
   2.2.5.2. Pulldown assay ........................................................................................44
   2.2.5.3. Transfer of biotin marker to cellular components .....................................44
   2.2.5.4. Western identification and analysis of proteins ........................................45
   2.2.5.5. Biosensor analysis of SSL11 interaction with sLe\(^x\) and sLacNAc ..........45
2.2.6. Interactions with cells .....................................................................................46
   2.2.6.1. SSL11 competition for neutrophil receptors .............................................46
   2.2.6.2. Neutrophil aggregation .........................................................................46
   2.2.6.3. Platelet aggregation and clotting .............................................................46
2.2.7. Functional analysis .........................................................................................47
   2.2.7.1. Complement Haemolytic Assay ...............................................................47
   2.2.7.2. Human complement ELISA ....................................................................47
   2.2.7.3. Activation of neutrophils ........................................................................48
   2.2.7.4. Cell death of neutrophils .......................................................................48
   2.2.7.5. Apoptosis of neutrophils .......................................................................48
   2.2.7.6. Calcium mobilisation in neutrophils .......................................................49
   2.2.7.7. Live cell imaging of neutrophils responding to S. aureus ......................49
2.2.8. Analysing SSL11 in cells ...............................................................................50
2.2.8.1. Cleavage of SSL11 in neutrophils ................................................................. 50
2.2.8.2. Subcellular fractionation of SSL11 in neutrophils ........................................ 50
2.2.8.3. Gelatin gels to test activity of gelatinase ....................................................... 50
2.2.8.4. Live confocal imaging of SSL11 internalisation .............................................. 51
2.2.8.5. Live imaging of organelle localisation ............................................................ 51
2.2.8.6. Chemical inhibition of SSL11 entry ............................................................... 51
2.2.8.7. Imaging of fixed specimens ........................................................................... 51
2.2.8.8. Transmission Electron Microscopy ................................................................. 52
2.2.9. Phylogenetic analysis ....................................................................................... 53
2.2.9.1. Protein sequence alignment .......................................................................... 53
2.2.9.2. Generation of SSL11 phylogenetic tree ......................................................... 53

Chapter 3. Analysing SSL11 interactions .................................................................... 54
3.1. Introduction ........................................................................................................... 54
3.2. Results .................................................................................................................. 54
3.2.1. Analysis of SSL11 from sequenced S. aureus ................................................... 54
3.2.2. Purifying SSL11 alleles .................................................................................... 57
3.2.3. SSL11 dimer isolation ...................................................................................... 60
3.2.4. Biasensor analysis of SSL11 binding sialylated carbohydrates ....................... 62
   3.2.4.1. SSL11 alleles binding sLe\(^x\) ....................................................................... 63
   3.2.4.2. SSL11\(_{US6610}\) monomer compared to dimer binding sLe\(^x\) ......................... 65
   3.2.4.3. Biacore analysis of SSL11\(_{US6610}\) binding sialylactosamine ...................... 66
3.2.5. Flow cytometric analysis of SSL11 binding neutrophils .................................. 68
   3.2.5.1. SSL11 competition for neutrophil receptors ............................................... 68
   3.2.5.2. Titration of SSL11 binding neutrophils ....................................................... 70
3.2.6. SSL11 induced aggregation of neutrophils ...................................................... 71
   3.2.6.1. Induction of neutrophil aggregation by different SSL11 alleles ............... 71
   3.2.6.2. Comparison of neutrophil aggregation by monomer and dimer SSL11 ....... 73
   3.2.6.3. Aggregation of mononuclear cells ............................................................. 74
3.2.7. SSL11 interaction with monocytes and macrophages ...................................... 75
   3.2.7.1. SSL11 internalisation into monocytes ......................................................... 75
   3.2.7.2. SSL11 internalisation into macrophages ..................................................... 76
3.2.8. Biotin labelling of SSL11 bound cellular components ...................................... 77
   3.2.8.1. Detecting biotin after incubation of Sulfo-SBED conjugated SSL11 with cells .................................................................................................................................. 78
3.2.8.2. Detecting the dimer of Sulfo-SBED SSL11 treated cells ......................... 80
3.2.8.3. SSL11 alleles forming dimers as detected by biotin transfer ................... 81
3.2.9. Pulldowns of cell lysates with the SSL11 alleles ...................................... 82
3.3. Discussion ........................................................................................................... 83

Chapter 4. Examining what effect SSL11 has on functional immune responses .......... 87
4.1. Introduction ............................................................................................................. 87
4.2. Results ................................................................................................................... 87

4.2.1. Analysis of SSL11 and neutrophil chemotaxis ................................................. 87
  4.2.1.1. SSL11 polarising to the neutrophil uropod during chemotaxis .................. 89
4.2.2. Neutrophil calcium signalling following activation ............................................ 90
4.2.3. Actin changes in activated neutrophils ............................................................. 92
4.2.4. Analysis of SSL11 on the viability of neutrophils .............................................. 93
  4.2.4.1. Induction of cell death in the presence of SSL11 ................................... 93
  4.2.4.2. Analysis of neutrophil apoptosis in the presence of SSL11 .................... 94
4.2.5. SSL11 and complement .................................................................................. 96
  4.2.5.1. Complement-mediated haemolysis of erythrocytes .................................. 96
  4.2.5.2. SSL11 in human complement ELISAs .................................................... 97
4.2.6. SSL11 and platelet coagulation .................................................................. 99
4.2.7. Analysis of SSL11 cellular activity by mass spectrometry ............................ 101
4.3. Discussion ......................................................................................................... 103

Chapter 5. Examining internalised SSL11 in myeloid cells .................................... 106
5.1. Introduction ......................................................................................................... 106
5.2. Results ............................................................................................................... 107

5.2.1. Internalised SSL11 in neutrophils ............................................................... 107
  5.2.1.1. SSL11 allele internalisation into myeloid cells .................................. 107
  5.2.1.2. SSL11 monomer internalisation into neutrophils ................................ 110
5.2.2. Modification of SSL11 within cells ............................................................ 111
5.2.3. Subcellular fractionation of granules from neutrophils treated with SSL11 ...... 112
5.2.4. Chemical inhibition of SSL11 entry into myeloid cells ............................... 115
  5.2.4.1. Inhibiting entry of SSL11 into neutrophils ....................................... 115
  5.2.4.2. Inhibiting entry of SSL11 into macrophages ...................................... 117
5.2.5. Neutrophil aggregation with chemical inhibition of SSL11 internalisation .... 118
5.2.6. Observing the interaction of SSL11 with the cytoskeletal network ............. 120
5.2.6.1. SSL11 trafficking along the microtubule network ........................................ 120
5.2.6.2. Observing SSL11 and actin ........................................................................ 122
5.2.6.3. Observing SSL11 and vimentin ................................................................. 123
5.2.7. Visualising SSL11 and cellular organelles ..................................................... 124
5.2.7.1. Visualising SSL11 and the late endosome .................................................. 124
5.2.7.2. Visualising SSL11 and the Golgi apparatus .............................................. 125
5.2.7.3. Visualising SSL11 and the endoplasmic reticulum .................................... 126
5.2.7.4. Visualising SSL11 and lysosomes ............................................................. 127
5.2.8. Transmission Electron Microscopy of SSL11 in neutrophils ......................... 128
5.3. Discussion .......................................................................................................... 130

Chapter 6. Discussion ............................................................................................... 133
6.1. Introduction ......................................................................................................... 133
6.2. Analysis of conservation of the SSL11 sequence .............................................. 134
6.3. Dimerisation of SSL11 ...................................................................................... 134
6.4. SSL11 binding sialylated receptors .................................................................. 136
   6.4.1. Gangliosides .................................................................................................. 136
6.5. Is there a specific receptor for SSL11? ............................................................. 137
   6.5.1. Macrophage – 1 antigen ............................................................................... 137
6.6. Parallels to the AB family of toxins .................................................................. 138
6.7. Future directions ................................................................................................. 139
   6.7.1. Characterisation of SSL11 interacting with Mac-1 ........................................ 139
   6.7.2. Examine SSL11 intracellular trafficking ..................................................... 139
   6.7.3. SSL11 interacting with the downstream hypothetical protein ..................... 140
   6.7.4. In vivo assays ................................................................................................ 140
6.8. Concluding remarks ........................................................................................... 140

Chapter 7. Appendix ................................................................................................. 141
7.1. SSL11 Sequence Alignment .............................................................................. 141
7.2. Plasmids ............................................................................................................ 143
7.3. Mass Spectrometry results .............................................................................. 144
   7.3.1. SSL11 .......................................................................................................... 144
   7.3.2. Vimentin ...................................................................................................... 145
   7.3.3. Keratin ........................................................................................................ 146
   7.3.4. Cytokeratin .............................................................................................. 147
7.3.5. Actin ......................................................................................................................... 148
7.3.6. Activation of neutrophils with fMLF ................................................................. 149
7.3.7. Myeloperoxidase assay ...................................................................................... 150
References.................................................................................................................... 151
List of Figures

Figure 1.1. Rapid evolution of antibiotic resistance ............................................................. 4  
Figure 1.2. Complement cascade ....................................................................................... 7  
Figure 1.3. Neutrophil recruitment cascade ........................................................................ 8  
Figure 1.4. Siglec activity in immune cell function ............................................................ 13  
Figure 1.5. Structure of Sialyl Lewis X .............................................................................. 14  
Figure 1.6. Comparing the structures of superantigens and SSLs ...................................... 22  
Figure 1.7. Phylogenetic tree of the SSL proteins and TSST .............................................. 23  
Figure 1.8. Crystal structures of SSL4, 5 and 11 binding sLe\textsuperscript{x} ......................... 27  
Figure 1.9. Alignment of SSL proteins belonging to the carbohydrate binding clade ....... 28  
Figure 1.10. SSL11 internalisation into neutrophils .......................................................... 29  
Figure 1.11. Structure of the SSL11 dimer ....................................................................... 31  
Figure 3.1. Representation of conserved amino acids in the structure of SSL11 ............... 55  
Figure 3.2. Maximum Likelihood phylogenetic tree of SSL11 ........................................... 56  
Figure 3.3. Alignment of SSL11 alleles used in this study .................................................. 57  
Figure 3.4. Anion exchange chromatography to purify SSL11 proteins ..................... 59  
Figure 3.5. Size exclusion separation of SSL11 monomers and dimers .............................. 61  
Figure 3.6. Biacore analysis of SSL11\textsubscript{US6610} R179A exhibiting no binding to sLe\textsuperscript{x} ................................................ 63  
Figure 3.7. Biacore analysis of SSL11 alleles binding BSA-sLe\textsuperscript{x} .......................... 64  
Figure 3.8. SSL11\textsubscript{US6610} dimer demonstrates prolonged association with sLe\textsuperscript{x} when compared to the monomer ................................................ 65  
Figure 3.9. Biacore analysis of SSL11\textsubscript{US6610} binding sLacNAc shows no difference when compared to binding sLe\textsuperscript{x} ........................................................................ 66  
Figure 3.10. No competition between fluorescein labelled - SSL11\textsubscript{US6610} and unlabelled SSL11\textsubscript{US6610} is observed showing SSL11 is able to bind multiple sialylated receptors ....... 69  
Figure 3.11. Titration of fluorescein labelled SSL11\textsubscript{US6610} binding neutrophils exhibited no saturation ................................................................................................................ 70  
Figure 3.12. Aggregation of neutrophils with SSL11 alleles .............................................. 72  
Figure 3.13. Neutrophil aggregation caused by SSL11 dimer ........................................... 73  
Figure 3.14. SSL11 aggregation of mononuclear cells and THP-1 monocytes .................. 74  
Figure 3.15. Live cell imaging of SSL11 internalisation into primary monocytes but not THP-1 cells .................................................................................................................. 75  
Figure 3.16. Live cell imaging of SSL11 internalisation into primary macrophages but not THP-1 derived macrophages ................................................................. 76  
Figure 3.17. 3D reconstruction of SSL11 internalised into primary macrophages ............ 77  
Figure 3.18. Sulfo-SBED biotin tagging of SSL11 binding to neutrophils ....................... 78  
Figure 3.19. Western blot of SSL11 biotin tagging of macrophages and THP-1 cells ........ 79  
Figure 3.20. Detection of SSL11 dimer in macrophages linked with Sulfo-SBED conjugated SSL11\textsubscript{US6610} .................................................................................................. 80  
Figure 3.21. SSL11 alleles dimerising on the surface of macrophages ............................. 81  
Figure 3.22. SSL11 allele pulldowns of granulocyte and mononuclear cell lysates .......... 82
Figure 4.1. SSL11 treated neutrophils show diminished surface detachment when migrating to opsonised *S. aureus*. ................................................................. 88
Figure 4.2. SSL11 concentrates to the uropod in activated neutrophils ......................... 89
Figure 4.3. Calcium mobilisation following activation of neutrophils is inhibited by CHIPS but not SSL11 ................................................................. 91
Figure 4.4. Activated neutrophils treated with SSL11 have altered actin when compared to non-treated cells ........................................................................ 92
Figure 4.5. SSL11 does not induce neutrophil apoptosis .............................................. 95
Figure 4.6. None of the SSL11 proteins have any effect on total complement activity .......... 96
Figure 4.7. SSL11 has no inhibitory activity in any of the human complement pathways .... 98
Figure 4.8. SSL11 does not alter coagulation of plasma ............................................. 100
Figure 4.9. Global analysis of neutrophil protein amounts ........................................... 101
Figure 4.10. Principle component analysis of SSL11 treated and untreated neutrophil protein amounts ........................................................................ 102
Figure 5.1. Internalisation of SSL11 into neutrophils .................................................. 108
Figure 5.2. Internalisation of SSL11 into primary macrophages .................................. 109
Figure 5.3. SSL11 monomer internalised into neutrophils .......................................... 110
Figure 5.4. Western blot demonstrates that SSL11 is not cleaved within neutrophils ....... 111
Figure 5.5. Western analysis of neutrophil subcellular fractionation of neutrophils containing SSL11 ........................................................................... 113
Figure 5.6. Gelatinase assay on neutrophil subcellular fractions demonstrates SSL11 is present in fractions also containing gelatinase ............................................. 114
Figure 5.7. SSL11 internalisation with endocytosis inhibitors demonstrates entry is via clathrin ......................................................................................... 116
Figure 5.8. Live cell imaging of primary macrophages incubated with SSL11 demonstrates that internalisation is an active process ........................................ 117
Figure 5.9. Chemical inhibition of SSL11 internalisation into macrophages is only observed with inhibitors of clathrin-dependent endocytosis .............................. 118
Figure 5.10. SSL11 induced neutrophil aggregation is less compact in the presence of clathrin inhibitors ......................................................................... 119
Figure 5.11. Live confocal imaging of SSL11 in primary macrophages with stained microtubules ......................................................................................... 120
Figure 5.12. Confocal imaging of SSL11 associating with microtubules in fixed primary macrophages ................................................................. 121
Figure 5.13. Confocal imaging of SSL11 and actin microfilaments showing no association ......................................................................................... 122
Figure 5.14. Confocal imaging of SSL11 and vimentin intermediate filaments showing no association ................................................................. 123
Figure 5.15. Confocal imaging of SSL11 inside late endosomes in fixed macrophages ... 125
Figure 5.16. Confocal imaging of SSL11 and the Golgi apparatus ................................ 126
Figure 5.17. Confocal imaging of SSL11 and the Endoplasmic reticulum ..................... 127
Figure 5.18. Confocal imaging of SSL11 and lysosomes .............................................. 128
Figure 5.19. Transmission electron microscopy of SSL11 in neutrophils .................... 129
List of Tables

Table 1.1 *S.aureus* components involved in immune evasion .................................................15
Table 2.1. Details on *ssl11* genes cloned into pET32a.3C .........................................................32
Table 2.2. Summary of antibodies used .........................................................................................33
Table 2.3. Making acrylamide gels for SDS-PAGE .................................................................38
Table 3.1. Properties of the SSL11 alleles ..................................................................................58
Table 3.2. Summary of calculated steady state affinity SSL11 dissociation constants ..........67
Table 4.1 Neutrophil cell death in the absence of presence of SSL11 .................................93
Abbreviations

SI prefixes

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

SI units

s | second
min | minute
h | hour
M | molar
g | gram
L | litre

Other units and abbreviations

°C | degrees Celcius
2D | two dimensional
3D | three dimensional
Abs | absorbance
ACD | Acid citrate dextrose
ADP | adenosine diphosphate
AMP | antimicrobial peptide
APS | Ammonium persulfate
ATCC | American type cell culture collection
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C5aR</td>
<td>C5a complement receptor</td>
</tr>
<tr>
<td>CC</td>
<td>Clonal Complex</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cfu</td>
<td>colony forming units</td>
</tr>
<tr>
<td>CHIPS</td>
<td>chemotaxis inhibitory protein of <em>S. aureus</em></td>
</tr>
<tr>
<td>CR1</td>
<td>complement receptor 1</td>
</tr>
<tr>
<td>cv</td>
<td>column volume</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycoltetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
</tr>
<tr>
<td>Fc</td>
<td>immunoglobulin crystallisable fragment</td>
</tr>
<tr>
<td>FcαR</td>
<td>myeloid immunoglobulin A Fc receptor</td>
</tr>
<tr>
<td>FcγR</td>
<td>myeloid immunoglobulin G Fc receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
</tbody>
</table>
Fig  
figure

fMLF  
N-formylmethionyl-leucyl-phenylalanine

FPLC  
fast performance liquid chromatography

g  
relative centrifugal force

GFP  
Green fluorescent protein

GlcNAc  
N-acetyl glucosamine

GPCR  
G-protein coupled receptors

GPI  
glycosylphosphatidylinositol

HBS  
HEPES buffered saline

HBSS  
Hanks balanced salt solution

HEPES  
4-(2-hydroxyethyl)piperazine-ethanesulfonic acid

HRP  
horse radish peroxidase

IDA  
iminodiacetic acid

Ig  
immunoglobulin

IL  
interleukin

IMAC  
immobilized metal affinity chromatography

IPTG  
isopropylthio-β-galactosidase

kb  
kilobase

K<sub>D</sub>  
dissociation constant

Da  
Dalton

LC/MS  
Liquid chromatography/mass spectrometry

LB  
Luria-Burtani

LPS  
lipopolysaccharide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>Mac-1</td>
<td>macrophage-1 antigen</td>
</tr>
<tr>
<td>MALDI-MS</td>
<td>matrix assisted laser desorption/ionisation-mass spectrometry</td>
</tr>
<tr>
<td>MHC II</td>
<td>major histocompatibility complex class II</td>
</tr>
<tr>
<td>MLST</td>
<td>Multi-locus sequence typing</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloprotease</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin-resistant <em>S. aureus</em></td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biotechnology Information</td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>OB</td>
<td>oligosaccharide/oligonucleotide-binding</td>
</tr>
<tr>
<td>O/N</td>
<td>overnight</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PPP</td>
<td>Platelet poor plasma</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
</tr>
<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>R&lt;sub&gt;eq&lt;/sub&gt;</td>
<td>response at equilibrium</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</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>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SET</td>
<td>staphylococcal exotoxin-like toxin</td>
</tr>
<tr>
<td>Sia</td>
<td>sialic acid – N-acetylneuraminic acid/ Neu5Ac</td>
</tr>
<tr>
<td>sLacNAc</td>
<td>sialyllactosamine/ Neu5Acα2-3Galβ1-4GlcNAc</td>
</tr>
<tr>
<td>sLe&lt;sup&gt;x&lt;/sup&gt;</td>
<td>sialyl Lewis X/ Neu5Acα2-3Galβ1-4(Fucα1-3)GlcNAc</td>
</tr>
<tr>
<td>SSL</td>
<td>staphylococcal superantigen-like protein</td>
</tr>
<tr>
<td>ST</td>
<td>sequence type</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N, N-teramethylethylenediamine</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)propane-1-3-diol</td>
</tr>
<tr>
<td>Thx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction

1.1. Overview

*Staphylococcus aureus* is one of the most common causative agents of both hospital-acquired and community-acquired infections. There is serious concern over the growing prevalence of both methicillin-resistant and vancomycin-resistant strains. Resistance to these antibacterial drugs has pressured the development of antibiotics that, at a minimum, will control the infection. Immune evasion is an important part of *S. aureus* pathology. Normally the host eradicates the bacteria through innate defences such as complement and neutrophil mediated cell killing. However, *S. aureus* evades these important immune defences by inhibiting components involved in chemotaxis, opsonisation, and bacterial damage. In addition, *S. aureus* is able to destroy immune cells. *S. aureus* is also able to ‘distract’ the adaptive immune system making it unable to produce memory resulting in possible recurrent infections. Superantigens are responsible for this phenomenon as they activate a subset of Vβ T cells that produce large doses of cytokines. A recent discovery identified staphylococcal superantigen-like (SSL) proteins that shared a conserved protein structure with the superantigens. Analysis showed that the SSLs however do not share the function of the superantigen, and were much more conserved in the genome of multiple strains. Characterization of SSL7 showed its importance in immune evasion as it binds both IgA and C5. A clade of SSLs has also been shown to bind sialylated molecules such as PSGL-1, an important molecule in leukocyte migration. Collectively, the SSLs appear to have important, non-redundant functions and so may be plausible targets for drug design.
1.2. Staphylococcus aureus

1.2.1. The organism

*Staphylococcus aureus* is a coccoid, Gram positive bacterium that was named after its characteristic gold pigmentation in culture [1]. Humans are a natural reservoir of *S. aureus* with primary colonisation being localized to the moist squamous epithelium of the anterior nares [1, 2]. *S. aureus* is also a known commensal of the vagina, axillae (underarm), pharynx and damaged skin surfaces [1]. Transient association is common with estimates ranging from 50% to 60% of the global population [1, 2]. Persistent colonisation is less common with only 20% to 30% of the population having a permanent association with *S. aureus* [1, 2].

1.2.2. Pathogenesis of *S. aureus*

Colonisation with virulent strains is the initial risk factor in the development of disease [1, 3]. Carriage of *S. aureus* within the nasal passages has shown to be a risk factor in the development of bacteremia (presence of bacteria within the blood) [3]. However mortality rates within these patients are reduced when compared to non-carriers [3]. This protective immunity is a likely consequence of the presence of antibodies that are targeted to the colonising strains [3, 4]. Disease normally manifests as superficial skin infections which include impetigo and some abscesses [1]. Inoculation of bacteria into other sites following mucosal or skin damage, can cause potentially fatal, serious invasive infections which occasionally result in: septic arthritis (joint infection); osteomyelitis (bone inflammation); endocarditis (infection of the inner heart lining); bacteremia and pneumonia [1]. Strains that possess certain toxin genes are able to cause toxic shock syndrome which is potentially fatal if left untreated [1, 2].

*S. aureus* is responsible for most cases of hospital-acquired (nosocomial) and community-acquired infections [1]. Transmission occurs through contact between infected patients, healthcare workers and wounded patients [1]. Devices, such as catheters, are another inoculation source as serum contaminants on the device accommodate *S. aureus* biofilms to develop as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) adhere to fibrin and fibrinogen in the serum [1]. Hospitals therefore require strict cleaning regimes to remove any source of potential *S. aureus* inoculum. Colonisation with *S.
Chapter 1 – Introduction

*aureus* increases the potential for subsequent infections and so virulent infections must be completely eradicated before recovery [1, 5].

### 1.2.3. Antibiotic resistance

The incidence of community acquired infections is increasing in many countries including New Zealand [1, 6-9]. Of more concern is the increasing incidence of methicillin-resistant *S. aureus* (MRSA) [6-8]. Initially, penicillin resistance strains were increasing, and methicillin resistance strains were confined to rare nosocomial infections [6-8]. However, the prevalence of methicillin resistance is increasing and are now commonly isolated from community-acquired infections and only about 5% of *S. aureus* strains remain sensitive to penicillin [1, 6-8]. Resistance to methicillin is conferred by the *mecA* operon that is located on a region of the genome known as the staphylococcal cassette chromosome SCCmeC [6-8]. *mecA* expresses the penicillin-binding protein 2a that provides resistance to methicillin and other β-lactams like penicillin [6-8]. Sequencing has shown that there are different types of SCCmeC providing some insight into the evolution of resistance; where types I, II and III are represented by most nosocomial strains and type IV being abundant (some exceptions) in community-acquired infections [6-8]. Currently there are eleven known variations of the SCCmeC cassette that vary in size and contain differing alleles of the *mecA* gene and *ccr* (cassette chromosome recombinase) genes responsible for the recombinational mobility of these genetic elements [7]. With penicillin being ineffective and the growing resistance to methicillin and other anti-microbial agents, vancomycin remains the last successful therapy for MRSA infections. However, in 1997, isolates from Japan were found to have reduced sensitivity to vancomycin and were annotated as vancomycin intermediate *S. aureus* (VISA) [10]. In 2002, an isolate completely resistant to vancomycin (VRSA) was discovered in the USA [11]. This strain was shown to have acquired a vancomycin resistance operon, *vanA*, from vancomycin resistant enterococci (VRE) that affects the biochemical makeup of the cell wall rendering vancomycin, a drug that incorporates into and blocks cell wall synthesis, ineffective [11, 12].
Chapter 1 – Introduction

The evolution of antibiotic resistance has been astonishingly fast with the resistant strains becoming prevalent within a few years of the introduction of the antibiotic (fig. 1.1) [8]. The mounting threat of *S. aureus* strains that resist all the effective antibiotics is increasing the demand for novel antibiotics that eradicate the infection, or at least prevent the onset of severe symptoms. The development of novel antibiotics will require an understanding of the factors and immune responses that contribute to the pathogenesis of the bacteria. With this knowledge, antibiotics can be designed to target important and conserved factors unique to *S. aureus* so that its pathogenesis can be attenuated.

![Figure 1.1. Rapid evolution of antibiotic resistance](image)

**Figure 1.1. Rapid evolution of antibiotic resistance**

The rapid increase in antibiotic resistance over the past century is associated with the large use of antibiotics. Following the use of penicillin, penicillin resistance was seen in *S. aureus* and was largely dominated by a strain known as Phage type 80/81. Penicillin, becoming an inadequate treatment, promoted the use of methicillin. Within years of clinical use, the first strain of methicillin resistant *S. aureus* (MRSA) was identified and contained SCCmecI. Evolution over the next twenty years produced the allotypes SCCmecII and III which resulted in large outbreaks in hospitals worldwide. The prevalence of community acquired MRSA (CA-MRSA) has continued to increase from the 1990s and is associated with the acquisition of SCCmecIV. With the use of vancomycin to treat MRSA, vancomycin intermediate resistance (VISA) and completely vancomycin resistant (VRSA) *S. aureus* have since emerged. From reference [8].
1.3. The host immune response to \textit{S. aureus}

If the bacteria are successful in penetrating the skin and mucosal barriers (nasal and gastrointestinal), the host's immune system is responsible for the removal of the bacteria from the tissues or blood. A successful immune response to clear \textit{S. aureus} infection is primarily performed by the innate immune system, especially through the action of neutrophils \cite{1, 2}. A co-ordinated series of events control the innate immune system to allow the passage of neutrophils from the circulatory system to the site of infection where they are able to contain and destroy the bacteria.

1.3.1. Attraction of neutrophils to the site of infection

1.3.1.1. Proinflammatory cytokines and chemokines

Tissue resident macrophages and dendritic cells that encounter \textit{S. aureus} are activated to produce proinflammatory cytokines such as tumor necrosis factor α (TNFα), following engagement of bacterial markers with the pattern recognition receptors (PRR) on immune cell surfaces \cite{1, 13}. TNFα is then able to activate other immune cells within the infection site along with adjacent endothelial cells. Endothelia are also activated by the damage caused by the invading bacterium and express PRR to recognise foreign bacteria \cite{1}. Activated endothelial cells, along with activated macrophages, produce interleukin-8 (IL-8), a chemokine that attracts neutrophils and monocytes \cite{1, 13}. IL-8 in endothelial cells are stored in Weibel-Palade bodies allowing for the immediate response to infection \cite{14}. This process is amplified by the eventual recruitment of neutrophils and monocytes to the site of infection whereby they are activated and produce proinflammatory cytokines like TNFα and IL-1β \cite{13}. Proinflammatory cytokines are essential in regulating and improving the activity of the recruited immune cells.
1.3.1.2. Complement

Complement is a family of important serum proteins, that when proteolytically cleaved, opsonise (C3b), destroy (membrane attack complex: C5b-9) or cause vasodilation and attract leukocytes (C3a and C5a) [2, 15-17]. Complement activation proceeds via one of three different pathways. These routes are known as the classical, lectin and alternative pathways and vary depending on the activation source (fig. 1.2) [15, 16].

The classical pathway is initiated by C1q binding antibodies complexed with antigens [15, 16]. A conformational change in C1q activates C1r and C1s in a calcium-dependent manner, forming a complex capable of cleaving C4 into C4a which is released and C4b which associates with the bacterial surface [15, 16]. C4b binds C2, which is also cleaved by the C1qrs complex into C2a, forming the C3 convertase (C4bC2a) [15, 16]. The lectin pathway creates the same C3 convertase but cleaves C4 and C2 in a different manner [15, 16]. Mannose binding lectin (MBL) binds terminal mannose residues on the bacterial cell surface [15, 16]. MBL then interacts with MBL associated serine proteases 1 and 2 (MASP1/2) to cleave C4 and C2 to generate the C3 convertase (C4bC2a) [15, 16]. The alternative pathway is different in that it uses the low rate of C3 spontaneous hydrolysis to generate C3b which recognises pathogen associated molecular patterns (PAMP) on bacterial surfaces [15, 16] C3b then binds factor B forming a complex which is recognised by factor D. Factor D cleaves factor B to form the alternative C3 convertase (C3bBb), which is stabilised by the co-factor properdin [15, 16]. From this point, the pathways are the same as the C3 convertases then cleave C3 to generate C3a and C3b. C3b associates with the convertases to form the C5 convertase (C4bC2aC3b or C3bBbC3b) [15, 16]. The C5 convertase cleaves C5 to generate C5a and C5b. C5b binds to the microbial surface and binds C6 and C7, allowing the subsequent binding of C8 and polymerised C9 [15, 16]. The C5bC6789 complex is known as the membrane attack complex (MAC) as it forms a cylindrical pore through the bacterial membrane resulting in its destruction [15, 16]. The MAC forms on the surface of gram positive bacteria, however it is unable to form a pore owing to their thicker peptidoglycan layer [2, 15, 16, 18]

While the membrane attack complex is unable to destroy the gram positive S. aureus, the opsonisation of S. aureus with C3b is essential in promoting and increasing the efficacy of phagocytosis [2, 13, 17, 19, 20]. The chemotactic proteins C3a and C5a are also essential in attracting phagocytes to the site of infection [13, 15, 16].
Figure 1.2. Complement cascade

The classical pathway involves complement C1, C2, C4 and bacterium specific antibodies. The lectin pathway involves bacterial specific lectins like mannose binding lectins (MBL). The alternative pathway involves factors B and D and endogenous bacterial structures that are recognised by C3. All 3 pathways form the C3 convertase that cleave C3 to C3b and C3a to continue in the pathway. *S. aureus* proteins that interfere with components of the pathway are shown in green. From reference [17].

The classical pathway links the adaptive and innate systems together as it requires anti-staphylococcal antibodies to initiate the pathway. However the precise roles of antibodies in *S. aureus* immunity remains elusive as antibody titres in patients do not always correlate with protection to subsequent infection (which probably reflects the manipulation of adaptive responses by *S. aureus* – see later) with the exception of antibodies to toxic shock syndrome toxin, TSST-1 [1, 4, 21].
1.3.2. Process of neutrophil migration

Following recognition of chemokines, neutrophils migrate from the blood stream to the site of infection using a strict progression of cellular events. In most tissues the recruitment of neutrophils is achieved by a cascade of cell tethering, rolling, adhesion, crawling and migration (fig. 1.3) [22]. The recruitment cascade is sequential and any perturbations in the process have pathologic consequences with diseases known as leukocyte adhesion deficiencies (LAD) [23].

![Neutrophil recruitment cascade](image)

Figure 1.3. Neutrophil recruitment cascade

A) Neutrophils are recruited from the blood by the initial attachment of selectins to the activated endothelia expressing selectin ligands. This binding allows the neutrophil to slow down against the flow of blood allowing firmer adhesion and rolling achieved by integrins binding their ligands. Neutrophils then migrate past the endothelia via paracellular or transcellular processes granting neutrophils access to the infected tissue. B) Intravital imaging techniques have allowed the in vivo processes to be examined as seen in the image containing labelled neutrophils. From reference [22].
1.3.2.1. Molecules involved in neutrophil adhesion

Neutrophil tethering and rolling is achieved by the dynamic binding and dissociation of selectins with their ligands. The primary selectins on endothelia are P-selectin (CD62P) and E-selectin (CD62E) which bind the leukocyte receptor P-selectin glycoprotein ligand-1 (PSGL-1) [22, 23]. P-selectin is rapidly mobilised from Weibel-Palade stores in endothelia in response to activation [22, 23]. E-selectin expression is up-regulated following activation and so is not immediately present on the endothelia surface [22, 23]. P-selectin binding PSGL-1 is largely responsible for tethering and rolling as E-selectin deficient mice do not have impaired neutrophil migration [24]. However, E-selectin binding aids in the firmer attachment of cells and re-organisation of receptors [25-27]. L-selectin (CD62L) is also able to bind PSGL-1 and is believed to not only direct homing to lymphoid organs, but also accommodate neutrophil binding to neutrophils that have attached to the endothelia [22, 28]. P-selectin is also expressed on platelets and accommodates neutrophil rolling over platelets that have bound the damaged or activated endothelia [29].

Firm adhesion is achieved by the β2-integrins lymphocyte function associated antigen-1 (LFA-1: CD11a/CD18) and macrophage-1 antigen (Mac-1: CD11b/CD18) [22, 23]. LFA-1 binds intercellular adhesion molecule-1 and -2 (ICAM-1, ICAM-2) on endothelia to create more stable adhesion [22, 23]. LFA-1 is constitutively expressed at high levels in an inactive state that has weak ICAM-1 binding [22, 23]. Following outside-in activation by chemokines such as IL-8 and CXCL1, LFA-1 undergoes a conformational change which has a higher affinity for ICAM-1 resulting in firm adhesion [23, 30, 31]. The conformational change is dependent on talin-1 and kindling-3 and is transient allowing for the regulation of adhesion [23, 30]. Mac-1 is also able to bind ICAM-1 and is responsible for a more stabilised tight adhesion and the crawling of the neutrophil to a suitable position on the endothelial vessel [22, 23]. Internal stores of Mac-1 are rapidly mobilised to the cell surface following neutrophil activation [30]. Mac-1 also adopts a higher affinity state following IL-8 stimulation which generates an outside-in signal via talin and Ras-related protein 1 (Rap1) to create a conformational change in Mac-1 [31-33].
1.3.2.2. Leukocyte diapedesis

Diapedesis is the process whereby leukocytes passage from the blood to the tissue through the endothelial vessel wall. Two routes of diapedesis are known: paracellular where cells move between two endothelia; and transcellular where cells migrate through an endothelial cell [22, 23]. Paracellular migration requires a gap between endothelia with a low basement matrix [22]. Engagement of leukocytes with endothelia activate the necessary processes involved in endothelia contraction to accommodate the paracellular migration of the leukocytes [23]. Platelet/endothelia cell adhesion molecule-1 (PECAM-1) and CD99 have been shown to be critical in paracellular diapedesis as they form homodimers between endothelia and leukocytes [23, 34, 35]. ICAM-1 engaging LFA-1 and Mac-1 maintains the firm adhesion of the leukocytes to endothelia [22, 23]. Junctional adhesion molecules (JAM) are also capable of binding leukocytes to enable leukocyte migration between the endothelia. JAM-A and JAM-C have been shown to bind LFA-1 and Mac-1 respectively [36, 37].

Little is known about transcellular diapedesis. Leukocyte binding to endothelia induces clustering of ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1) which is required for transcellular diapedesis [38]. The endothelial cell membrane then protrudes and surrounds the leukocyte to form ‘domes’ that are rich in actin [38]. Internalisation of the leukocyte is dependent on caveolin and forms a vesiculo-vacuolar organelle [23, 38]. Actin is important in the dome formation and passaging the internalised cell with the actin binding leukocyte-specific protein 1 (LSP-1) being shown to be necessary in this process [39].

1.3.2.3. Lymphocyte polarisation

Neutrophil signalling following selectin binding as well as cytokine and chemokine activation drives the polarisation of neutrophils [40, 41]. Polarisation is essential in clustering receptors required for the sequential binding steps of neutrophil recruitment. Neutrophils polarise with a front leading edge known as the pseudopod and the tailing end known as the uropod. The pseudopod which senses the environment is rich in receptors such as GM3 gangliosides, chemokine receptors, phosphatidylinositol 3 kinases (PI3K), polymerised actin and the Rho proteins that regulate actin dynamics [40-43]. The uropod is involved in controlling the attachment and detachment of cells during migration and is rich in receptors such as GM1 gangliosides, Mac-1, PSGL-1 and L-selectin [31, 42, 43].
1.3.3. Neutrophil killing of bacteria

1.3.3.1. Phagocytosis and intracellular bactericidal processes

Phagocytosis is the co-ordinated process in which bacteria are engulfed by phagocytes such as neutrophils. Phagocytosis is enhanced by the opsonisation of bacteria with antibodies and/or complement C3b [20]. The antibody fragment crystalline (Fc) receptors are therefore critical in initiating phagocytosis. Ligation and clustering of the Fc receptors activate Src-family kinases which phosphorylate the Fc receptor allowing tyrosine kinase Syk and PI3K to interact with the receptor [20]. These kinases regulate Rho GTPases which control actin polymerisation that form membrane protrusions that engulf the bacteria [20]. Mac-1 is also important as it is the receptor for C3bi, a cleavage product of the C3b opsonin [20, 44]. Mac-1 and Fc receptors have been shown to work in concert to enhance phagocytosis and neutrophil activities [45-47]. The ingested bacteria are enclosed in phagosomes which undergo acidification and maturation. Neutrophils have a variety of methods with which to kill the contained bacteria. NADPH oxidase forms on the phagosome surface and catalyses the NADPH-dependent generation of superoxide (\(2\text{O}_2^{-}\)) which then reacts with, and damages the bacteria [48, 49]. Superoxide dismutase regulates the process by converting the superoxide to hydrogen peroxide (\(\text{H}_2\text{O}_2\)) [49]. The phagosome can also fuse with azurophilic and specific granules. Azurophilic granules contain myeloperoxidase (MPO) which catalyses the reaction of chlorine and hydrogen peroxide to produce hypochlorous acid (\(\text{ClO}^-\) a potent microbicide) and more hydroxyl radicals [48, 49]. Azurophilic and specific granules also contain an array of antimicrobial polypeptides (AMP) such as lysozyme, defensins and bactericidal/permeability increasing protein (BPI) that damage the bacterial membrane along with proteases such as elastase and proteinase-3 [48, 50]. Finally, the phagosome fuses with the lysosome which contains acid hydrolases that destroy the bacteria facilitating antigen presentation on the surface of the antigen-presenting cells (APC) [48, 49].

1.3.3.2. Extracellular killing mechanisms

The antimicrobial polypeptides are also secreted following neutrophil degranulation. An important AMP is lactoferrin, which sequesters iron [48, 50]. Iron is vital to the metabolism of \(S.\ aureus\) and its deficiency due to lactoferrin starves the bacteria. Neutrophils also release DNA nets known as neutrophil extracellular traps (NETs) which are coated with AMPs such
as elastase and MPO [48, 49, 51]. NETs are believed to trap microbes by electrostatic attraction and represent a concentrated and confined region where AMPs can function [51].

1.3.4. Importance of sialic acid in the immune system

Sialylation is an important glycosylation of lipids and proteins in deuterostomes and some microorganisms [52, 53]. There are approximately 50 known sialic acids (Sia) with the most common being N-acetylneuraminic acid (Neu5Ac), N-glycolyneuraminic acid (Neu5Gc) and N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac2) [52-54]. However humans do not express Neu5Gc owing to a loss in the cytidine monophosphate-N-acetylneuraminic acid hydroxylase enzyme responsible for the conversion from Neu5Ac to Neu5Gc [54]. Sia are added to terminal sugars using α2-3, α2-6 or α2-8 linkages, which is important for defining the specificity of molecules that recognise Sia sugars [52-54]. Sialylation is ubiquitous and occurs in many tissues but is of particular importance to the immune system and is involved in seemingly opposite roles.

Firstly sialylation of terminal sugars hides these sugars from immune recognition and designate a molecule as ‘self’ [52, 53]. Sia attached to galactose has been shown to prevent recognition of galactose by galactose binding receptors, such as galectins which are responsible for sequestering cells and inducing apoptosis [52, 53]. Sialylation of IgG has been shown to reduce its affinity for the Fcγ-receptors [55]. Factor H has also been shown to bind Sia coated molecules considered as ‘self’ which allows binding of Factor I which cleaves C3b and regulates the natural, low level cleavage of C3 [56]. It is also hypothesised that over-expression of Sia on the cell surface creates a net negative charge which creates electrostatic repulsion with other cells altering their potential interactions [53]. Bacteria such as Neisseria meningitidis and Group B Streptococci utilise Sia to mask the bacteria from the immune system [56, 57].

Conversely, Sia are an important component of immune recognition. Sialic-acid-binding immunoglobulin-like lectins (Siglecs) regulate immune cell adhesion and signalling events (fig. 1.4) [58]. For example Siglec-1 on macrophages binds to Sia on neutrophils and Siglec-2 (CD22) on B cells inhibits and regulates B cell activity and survival [58]. Siglecs contain or associate with immunoreceptor tyrosine-based inhibitory motifs (ITIM) and so are important regulators of excessive leukocyte activity [58]. Specificity is achieved by the structure of the Sia with CD22 Siglecs recognising α2-6 linked sugars, whereas CD33 related Siglecs can
recognise a variety of Sia modifications [58]. Bacteria that contain Sia can engage these receptors to inhibit immune activity. For example Group B Streptococci engage Siglec-9 on neutrophils to inhibit their activity [57].

Figure 1.4. Siglec activity in immune cell function

Leukocyte Siglec activity shown where engagement with Sia is involved with decreased: cellular activation; proliferation; adhesion; endocytosis; degradation and IFNα secretion or increased apoptosis or activation. Following engagement of Sia, ITIMs are phosphorylated by SRC kinases which recruit SH2- (SRC homology 2) domain-containing protein tyrosine phosphatase 1 and 2 as well as the suppressor of cytokine signalling 3 (SOC3) which regulate cellular activity. Siglec-H and Siglec-14 do not contain a cytoplasmic ITIM and associate with DAP12. DAP12, in contrast, contains an immunoreceptor tyrosine-based activation motif (ITAM) and recruits spleen tyrosine kinase (SYK) to activate cells. From reference [58].

Sialyl Lewis X (sLe\(^\alpha\)) is an important and ubiquitous blood antigen. It is an α2-3 Sia linked to galactose which contains a single fucose and N-acetylglucosamine (Neu5Aca2-3Galβ1-4(Fucα1-3)GlcNAc) (fig.1.5) [54]. The selectins involved in leukocyte recruitment and lymphoid tropism bind sLe\(^\alpha\) which are highly expressed on their target receptors such as PSGL-1 [22, 23, 52, 53, 59]. Sia appears to convey a negative charge for recognition with the α2-3 linkage to galactose being critical. Modifications that do not alter the α2-3 linkage (such as addition of a sulfate ester at C3 on galactose) have no effect on selectin binding [53, 60].
Chemical structure of sLe\(^\text{x}\) which contains the sialic acid (Sia) linked to galactose (Gal) via an α2-3 linkage. Galactose is linked by a β1-4 bond to N-acetylglucosamine (GlcNAc) which is further bonded to fucose (Fuc) by an α1-3 linkage. Adapted from reference [54].

**Figure 1.5. Structure of Sialyl Lewis X**

1.4. Immune evasion by *S. aureus*

1.4.1. Immune evasive virulence factors

*S. aureus* has evolved a large assortment of secreted and surface virulence factors that contribute to the evasion of immune detection and destruction (summarised in table 1.1). These virulence factors do not work discretely, but rather act together to alter the immune responses at different stages of infection [2]. Regulation is therefore important and several factors have been shown to control the differential expression of virulence components. These include quorum sensing, *agr*, *saeRS*, *srrAB* and σ\(^B\) [7, 61]. The toxins also show great strain specificity and probably contribute to the differences between nosocomial and community-acquired strains [2, 7]. Some examples of toxins that act on different components of the immune system are highlighted below.
Chapter 1 – Introduction

Table 1.1 *S. aureus* components involved in immune evasion

Bacterial surface proteins generally aid in immune evasion by decreasing accessibility of immune proteins to the bacterial antigens. Components involved in specific and controlled immune evasion are mostly secreted exotoxins that degrade proteins, nucleic acids and disrupt lipid membranes.

<table>
<thead>
<tr>
<th>Virulence Factor</th>
<th>Bacterial Location</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein A</td>
<td>Surface</td>
<td>Binds IgG incorrectly blocking recognition of IgG</td>
</tr>
<tr>
<td>Clumping Factor A</td>
<td>Surface</td>
<td>Binds fibrinogen</td>
</tr>
<tr>
<td>Polysaccharide capsule</td>
<td>Surface</td>
<td>Hinders opsonin binding</td>
</tr>
<tr>
<td>Chemotaxis inhibitory protein</td>
<td>Secreted</td>
<td>Acts as antagonist to neutrophil receptors C5aR and FPR</td>
</tr>
<tr>
<td>Staphylokinase</td>
<td>Secreted</td>
<td>Activates plasminogen to plasmin – degrades proteins</td>
</tr>
<tr>
<td>Extracellular adherence protein</td>
<td>Secreted</td>
<td>Binds ICAM-1 and TCR (anergy)</td>
</tr>
<tr>
<td>Extracellular fibrinogen binding protein</td>
<td>Secreted</td>
<td>Binds C3</td>
</tr>
<tr>
<td>Aureolysin</td>
<td>Secreted</td>
<td>Cleaves defensins</td>
</tr>
<tr>
<td>Panton-Valentine Leukocidin</td>
<td>Secreted</td>
<td>Damages neutrophils &amp; macrophages</td>
</tr>
<tr>
<td>Haemolysins</td>
<td>Secreted</td>
<td>Damages blood cells – neutrophils</td>
</tr>
<tr>
<td>Enterotoxins</td>
<td>Secreted</td>
<td>Links TCR to MHC II to generate abnormal response</td>
</tr>
<tr>
<td>Toxic shock syndrome toxin</td>
<td>Secreted</td>
<td>Links TCR to MHC II to generate abnormal response</td>
</tr>
<tr>
<td><em>Staphylococcus</em> complement inhibitor</td>
<td>Secreted</td>
<td>Binds C3 convertase</td>
</tr>
<tr>
<td>Coagulase</td>
<td>Surface</td>
<td>Converts fibrinogen to fibrin</td>
</tr>
</tbody>
</table>

1.4.1.1. Virulence factors that inhibit the chemotaxis of neutrophils

The most abundant chemoattractants in bacterial infections include complement proteins C3a and C5a as well as bacterial formylated peptides. Unlike eukaryotes, bacterial protein translation is initiated by formylated methionine and so is a characteristic of bacterial peptides [62-64]. Sixty percent of *S. aureus* strains secrete a protein called chemotaxis inhibitory protein of staphylococci (CHIPS) which binds both C5a- and formyl peptide-neutrophil receptors (C5aR and FPR) thus preventing the binding of their normal chemoattractive ligands, inhibiting migration [62-65]. CHIPS is a 14.1kD protein encoded by a prophage element called the immune evasion cluster (IEC) which contains many other virulence factors involved in immune evasion [63, 64]. Expression of this protein immediately occurs in early growth phase to prevent neutrophils accessing the site of
infection [64]. Experiments also demonstrated minor roles of CHIPS in neutrophil activation and phagocytosis [64]. CHIPS has two distinct domains with critical phenylalanine residues: one domain for binding C5aR, and the other for binding FPR [65, 66]. CHIPS has shown higher specificity for the two human receptors, when compared to mice [62-64]. In vitro and in vivo studies show significant neutrophil migration changes in dosage-dependent levels when compared to strains lacking CHIPS [62-64]. Subsequently a molecule that has been shown to also inhibit FPR and FPR-like 1 (FPRL1) dependent chemotaxis, has been designated as FPRL1 inhibitory protein (FLIPr) [67]. A homologue of FLIPr (FLIPr-like) sharing 73% identity has been shown to inhibit chemotaxis to a similar level as CHIPS [68].

The extracellular adherence protein (EAP, formerly known as MHC Class II analogous protein – MAP) is another secreted protein from *S. aureus* that inhibits leukocyte migration [69]. EAP binds intercellular adhesion molecule 1 (ICAM-1) on vascular endothelial cells and inhibits trafficking across these cells [69]. EAP blocks the normal interaction between ICAM-1 and LFA-1 and thus prevents diapedesis [69]. EAP has also been shown to be important in the internalisation of *S. aureus* in keratinocytes [70]

1.4.1.2. Virulence factors that inhibit opsonisation and phagocytosis

*S. aureus* produces capsules of differing serotype which reduce the affinity of opsonins to their targets due to the spatial interference of the capsule masking the surface antigens [2, 7, 71]. If any opsonins manage to bind to the bacterium, the capsule may also interfere with its recognition by the opsonin receptor [2].

The endoprotein clumping factor A (ClfA) binds fibrinogen through its N terminus and polymerises with two other ClfA through its C terminus [72]. The captured fibrinogen acts as a scaffold for IgG and complement to activate platelet aggregation [73]. Unlike other endoproteins which are expressed during exponential growth, ClfA is expressed during stationary phase by the σB promoter [74]. It is postulated that the fibrinogen coat and platelet aggregate reduces the accessibility of cells to the enclosed bacteria [2, 73]. ClfA has also been shown to bind complement Factor I which cleaves and inactivates C3b and C4b [75]. Removal of ClfA binding fibrinogen has been associated with increased host survivability in a bacteremia model [76].
Chapter 1 – Introduction

Protein A is one of the earliest and best characterised surface expressed virulence factors of *S. aureus*. It is a five-domained endotoxin that potently inhibits opsonisation [2, 71]. Protein A binds IgG Fc thereby orienting the antibody in the incorrect position preventing the interaction of FcγRIII with the Fc region [2, 77]. Crystallisation and site-directed mutagenesis confirmed this interaction following the observation that Protein A mutants are more susceptible to phagocytosis [2]. Surface immunoglobulin-binding protein (Sbi) has been identified as another protein capable of binding IgG preventing effective opsonisation [78].

*S. aureus* also produces several exotoxins to combat opsonins and phagocytosis. *Staphylococcus* complement inhibitor (SCIN) binds the C3 convertase and inhibits its function of cleaving C3 to C3b and C3a and thus prevents the accumulation of C3b on its surface [64, 79, 80]. This is achieved by stabilising the C3 convertase which prevents its intrinsic decay which is required for C3 cleavage [79, 80]. The absence of C3b therefore prevents the efficient phagocytosis of bacteria by neutrophils. SCIN is a 9.8kD protein that is encoded in the immune evasion cluster 2 (IEC2) and like CHIPS, is expressed immediately during the early growth phase [64]. Of the *S. aureus* strains tested, 90% have a functional *scin* gene [64, 79]. Experiments also showed SCIN had minor activities in controlling chemotaxis [79]. SCIN is not the only factor that acts on C3. *S. aureus* produces a 19kD protein called the extracellular fibrinogen-binding protein (Efb) [81]. Efb binds C3 and thus prevents its interaction with C3 convertase and therefore reduces the amount of C3b able to bind to the bacteria [79, 81]. Efb is constitutively expressed and so may be more important for persistent bacteria colonisation compared to chronic infections [79].

*S. aureus* also has a mechanism to destroy any opsonins that manage to attach to their surface. Staphylokinase (Sak) is an exotoxin that binds and converts plasminogen to plasmin – a potent serine protease that degrades antibodies and C3b attached to bacteria in close proximity to plasmin, as well as fibrin which acts to contain the bacteria in a clot [82]. Plasminogen is presented to Sak through the co-operative activities of Sbi and Efb [83]. Sak is another toxin encoded by the IEC2 [82].
1.4.1.3. Virulence factors that prevent damage

Defensins are primitive AMPs that disrupt microbial cell membranes damaging the bacteria. *S. aureus* secretes Sak and aureolysin to inhibit the activity of defensins [2, 71]. Sak has a high affinity binding domain for α-defensin [84]. Sak therefore prevents α-defensin from reaching the bacteria. Aureolysin is a metalloprotease that cleaves an antistaphylococcal human defensin – cathelicidin LL-37 [85]. Strains producing these exotoxins show significant resistance to defensins *in vitro* and *in vivo* compared to mutant strains [2, 84]. In addition, the chemical composition of the teichoic acids in the *S. aureus* cell wall prevents some cationic AMPs from binding. D-alanine substitution in teichoic acids neutralise their negative charge preventing the cationic AMP recognising the bacterial surface [86]. The presence of an O-acetyltransferase in the membrane of *S. aureus* prevents the action of lysozyme, a bactericidal protein, by modifying its ligand – muramic acid [87].

If any bacteria are engulfed by neutrophils, *S. aureus* produces proteins to protect it from the damaging reactive oxygen species (ROS). *S. aureus* produces two different superoxide dismutases (SOD) which convert the harmful superoxide to hydrogen peroxide (which is subsequently converted to oxygen and water) and oxygen [88]. Manganese is a required cofactor for SOD, and has been shown to rescue mutant SOD which are otherwise less virulent compared to wild type [88]. Interestingly, the carotenoid yellow pigment (Staphyloxanthin) has also been implicated in ‘soaking up’ ROIs [2, 89].

*S. aureus* is increasingly being found within host cells such as epithelia, endothelia, fibroblasts, osteoblasts, keratinocytes and neutrophils and so remain hidden to the immune system [70, 90-92]. Currently, the most important virulence factors in regulating *S. aureus* internalisation into host tissue are the fibronectin binding proteins A and B (FnBPA and FnBPB) [90, 92]. FnBPA/B binds fibrinogen and fibronectin which bridges their interaction with α5β1 integrins [90, 92]. This induces F-actin reorganisation to form a ‘zipper type’ engulfment of the bacteria [90, 92]. Entry into cells via this mechanism avoids the phagosome related damaging mechanisms and also hides the bacteria from all extracellular immune defence mechanisms. An intracellular life is enhanced by the finding that *S. aureus* is able to form small colony variants (SCV) which are metabolically slow and rich in virulence factors [5, 93, 94]. Intracellular *S. aureus* are important clinically as they represent a potential reservoir of bacteria that are secluded from antibiotics and may be the source of recurrent infections.
1.4.1.4. Virulence factors that destroy immune cells

*S. aureus* produces haemolysins (α, β, γ and δ) and several leukocidins that disrupt the integrity of blood cell membranes, thus destroying the cells [2]. The mechanism is via the insertion of a β-barrel pore into the membrane which disrupts cellular homeostasis and osmolarity (with the exception of β haemolysin which directly hydrolyses the plasma membrane lipid, sphingomyelin) [95]. The haemolysins are encoded by *hl* genes, and are under the control of the *agr* regulator with expression late in the growth phase [95, 96]. Phenol soluble modulins (PSM), which includes δ haemolysin, are a newly discovered family of pore forming molecules that are believed to not use a protein receptor [96]. PSMs have been shown to lyse neutrophils following the phagocytosis of *S. aureus* [97]. PSMs are also important in the structure of biofilms [98].

The bicomponent toxins (for example: γ haemolysin and Panton-Valentine leukocidin – PVL) are able to damage neutrophils and macrophages [96]. They are different to the other haemolysins because they are not synthesized as a monomer, but rather two subunits (class S and F) that assemble in leukocyte membranes [96]. *In vitro* analysis shows these toxins are able to destroy leukocytes as observed through a microscope [96]. Gamma haemolysin is produced by 90% of *S. aureus* strains, whereas the prophage that encodes PVL is only found in 2% of strains [2, 96]. Although PVL is not found in many strains, there seems to be a strong association of PVL in community acquired MRSA [96]. Although this association is under much debate as conflicting infection models have shown some or no association with PVL and virulence [6].

1.4.1.5. Virulence factors that modulate the adaptive immune system

*S. aureus* exhibits effective control over adaptive immune pathways as an apparent lack of memory cells accommodates subsequent infections. EAP has a 30 residue motif that is homologous to the peptide binding groove of MHC II [99]. EAP is able to interact with T cell receptors, alter their activity, and possibly make them anergic [100]. EAP deficient bacteria are less virulent in mice that had a dominant TH1 response [100]. When compared to wild-type, EAP deficient bacteria seemed to have a role in converting a TH1 response to a TH2 response [100].
Protein A is also known as a B cell superantigen and is able to bind the V\textsubscript{H}3 region of IgM exposed on B cells \[101\]. Binding to IgM activates the B cell but with an absence of costimulatory molecules, the cells undergo apoptosis instead \[101\]. The most important \textit{S. aureus} components in adaptive attenuation are the superantigens which are discussed in detail below. All these toxins deplete a significant repertoire of lymphocytes that would ordinarily be an important portion of the adaptive response to \textit{S. aureus}.

### 1.4.2. Superantigens

Superantigens (Sags) are secreted, potent T cell mitogens \[102-105\]. Sags are able to activate up to 20% of the T cell population with some only requiring picogram per mL quantities \[102-105\]. This is a vast number of activated T cells, when compared to normal antigen presentation which activates 1 naïve T cell in a population of \(10^5\)-\(10^6\) cells. The many activated T cells secrete cytokines, which also induce the production of cytokines from other leukocytes, resulting in vast quantities of cytokines (cytokine storm) which produce the symptoms of toxic shock \[102, 104, 105\]. These cytokines are mostly proinflammatory and include TNFa, IFNy, IL-1\(\beta\), IL-2, and IL-6 \[102, 104, 105\]. While most of the Sags are found in \textit{S. aureus} and \textit{Streptococcus pyogenes}, there is a growing list of Sags that have been discovered in other bacteria and viruses such as \textit{Yersinia pseudotuberculosis}, Epstein-Barr virus and Mouse mammary tumour virus \[104, 105\]. The Sags encoded by \textit{S. aureus} include the staphylococcal enterotoxins (SE) and toxic shock syndrome toxin (TSST) \[102, 103\]. The Sags encoded by \textit{S. pyogenes} include: streptococcal pyrogenic exotoxins (SPE), streptococcal superantigen (SSA) and streptococcal mitogenic exotoxins (SMEZ) \[105\].

#### 1.4.2.1. Structure of the superantigens

Modelling the evolution of the Sags is complex as the protein sequence similarity can vary from 15.5% to 90% \[104, 105\]. The general PROSITE sequence signature for the Sags is: K-X(2)-[LIVF]-X(4)-[LIVF]-D-X(3)-R-X(2)-L-X(5)-[LIV]-Y \[105\]. Evolution and conservation among the Sags is much more apparent at the structural level. The staphylococcal and streptococcal Sags adopt a similar structure with two distinct domains \[106, 107\]. The N terminal domain adopts a five-stranded \(\beta\)-barrel topology which is similar to the oligosaccharide/oligonucleotide-binding fold (OB-fold). The OB-fold has a binding face that has \(\beta\)-strands 2 and 3 at its centre and is surrounded by three loops that have sequence variation \[108, 109\]. This face accommodates large macromolecules and so many
ligands for OB-folds have been discovered and include oligosaccharides, oligonucleotides and proteins [108, 109]. The OB fold is present in organisms ranging from Archaea to mammals and may even have catalytic activities [108, 109]. The C terminal domain of Sags is composed of a long amphipathic α-helix packed against a β-sheet. This topology is known as the β-grasp fold. The two domains are linked by a solvent accessible α-helix.

1.4.2.2. Function of the superantigens

Sags activate T cells by binding both T cell receptors (TCR) and major histocompatibility complex II (MHC II) on antigen presenting cells [102-105]. The ultimate function of the Sags is to bring the TCR and MHC II molecules in close proximity so that their interaction will activate the T cell. There is a variation in affinity for different MHC II molecules as dictated by the sequence of the Sag, for example SEC2 preferentially binds HLA-DQ compared to HLA-DR [102]. The interaction between Sags and the TCR are specific with each Sag exhibiting a Vβ specificity (for one or more) [102]. No structure detail is available for the complete complex formed between the TCR, Sag and MHC molecules. Functional and thermodynamic data has suggested that there are three interactions that stabilize the complex: MHC with Sag, TCR with Sag and TCR with MHC [102, 104, 105]. The ultimate goal of the Sag is to form this complex so that the subset of Vβ T cells bound will become activated. Co-stimulatory molecules such as LFA-1, ICAM-1 and CD28 are necessary [110].

The most plausible benefit Sags may provide *S. aureus* is that the activation of a vast number of T cells subverts their normal regulation and distracts them from their target [2]. The adaptive immune system boasts specificity against antigens. So the Sag activation of 20% of the total T cell population abolishes this specificity as the majority will not recognize *S. aureus* as the usual presentation of *S. aureus* antigen is bypassed. In order to control the hyperproliferation of T cells, these T cells will need to be stopped by inducing their anergy. If any of these T cells were *S. aureus* specific, presentation of staphylococcal antigen to them will not elicit a response. Anergy in memory T cells was demonstrated in mice where blockages in cell signalling molecules produced the anergy in response to SEB [111]. The co-stimulatory molecule CTLA-4 has also been shown to induce anergy following SEA treatment, and that anti-CTLA-4 antibodies prevent this anergy [112].
1.4.3. Staphylococcal Superantigen-Like Proteins

Staphylococcal Superantigen-Like proteins (SSLs) are a family of 14 exotoxins that were discovered following homology searches to identify Sags [113, 114]. These proteins all contain the Sag PROSITE signature sequence and had an average sequence similarity of 25% with TSST-1 and SPE-C [113, 114]. The SSLs were previously known as Staphylococcal exotoxin-like proteins (SET) but were renamed following the International Nomenclature Committee for Staphylococcal Superantigens to standardise naming [115]. The SSLs are named after the Sags as they share not only the signature sequence, but also the conserved structure of an OB-fold connected to a β-grasp via a solvent accessible α-helix (fig. 1.6) [116-119]. However, global sequence alignments indicated poor sequence similarity with the Sags with an average identity commonly below 25% [102]. The SSLs also do not simultaneously bind MHC II and the TCR to activate T cell expansion [113, 114, 116, 120]. Instead, the SSLs have utilised the conserved OB-fold and β-grasp domains to create multiple different binding sites that are able to recognise different immune molecules (fig. 1.6).

Figure 1.6. Comparing the structures of superantigens and SSLs

The structure of the toxic shock syndrome toxin (TSST) superantigen is shown with the conserved N-terminal OB-fold (orange) and C-terminal β-grasp (blue) domains. The MHC and TCR binding sites for TSST are shown with key residues highlighted. SSL7 and SSL11 adopt a similar structure but have differing binding sites. SSL7 binding IgA is through residues in the OB-fold whereas C5 binding is performed by residues in the β-grasp domain. SSL11 binds the carbohydrate sLacNAc in a V-shaped depression formed on the β-grasp domain. Adapted from reference [102].
Chapter 1 – Introduction

The SSLs are expressed during colonisation and infection as antibodies directed against several of the SSLs have been detected in patients [120, 121]. These include antibodies directed to SSL2, 4, 5, 7, 8, 9, 10 and 11 from both healthy and infected donors [120-122]. Phylogenetic analysis of the SSLs show that the family divides into two distinct clades (fig. 1.7) [123]. These clades represent their immune evasive functions with the carbohydrate-binding and immune-modulatory molecules discussed below.

![Figure 1.7. Phylogenetic tree of the SSL proteins and TSST](image)

Figure 1.7. Phylogenetic tree of the SSL proteins and TSST

Bootstrap phylogenetic tree showing amino acid homology among the SSLs and TSST. Major clades are highlighted. SSLs that share amino acid that are required for carbohydrate binding are shown in the dashed box. SSLs that are immuno-modulating are shown in the black box and the SSLs in the immune evasion cluster 2 are shown in the grey box. From reference [123].
1.4.3.1. Genomic organisation and regulation of the ssl family

The SSLs are named in ascending order according to the position of the gene from the origin of replication, and is based on homology with strain MW2 which contains the full complement of ssl genes [115]. The first 11 ssl genes are clustered in a 19kb region that is located on a staphylococcal pathogenicity island (SaPIn2/SaPIm2/vSaα in the strains N315, Mu50 and MW2 respectively) [124-126]. The first 10 ssl genes lie in a contiguous region while ssl11 is separated from ssl10 by the type IC restriction modification genes hsdM and hsdS [124, 125, 127]. Each ssl gene has its own ribosomal binding site and promoter so are expressed individually with the exception of ssl11, which is co-expressed with an adjacent hypothetical gene downstream of the ssl11 gene [128, 129]. The ssl12, ssl13 and ssl14 genes are located 700kb downstream in immune evasion cluster 2 (IEC2) along with other genes encoding virulence factors such as Efb, extracellular complement-binding protein, SCIN homologues and FLIPr [130]. Initially there were twenty-six identifiable ssl alleles but following sequence alignment, this number was reduced to fourteen distinct ssl genes based on the observation that several S. aureus strains expressed allelic variants of the same ssl gene [120]. Global sequence alignments indicate that the SSLs share 36-67% identity [113]. Not all strains contain the full set of SSLs and PCR-RFLP (restriction fragment length polymorphism) indicated diversity in these genes (32). The strain variations and polymorphisms suggest that strong selection pressures are applied to their function in the pathogen host interaction [116, 120].

Quantitative real time PCR indicated that maximum expression of the ssl genes occur at stationary phase [114, 120]. Microarray analysis revealed that both SSL7 and SSL11 are regulated by the two component system (TCS), S. aureus exoprotein SaeRS [131]. It has been subsequently shown that the response regulator SaeR works in concert with the transcription factor, repressor of toxins (Rot), to bind the ssl promoter to induce transcription [132]. Both regulators are required and represent the first example where these regulators work in synergy [132]. Rot and SaeRS had previously been considered to work in opposition as virulent factors, such as cytotoxins, were upregulated by SaeRS but repressed by Rot [129, 131, 132]. It has also been shown that bacterial membrane damage increases the production of SSLs [129, 133]. This has been demonstrated as a consequence of membrane damage releasing Rot from repression by the RNA regulator, RNAIII [129]. RNAIII is expressed following Agr activation and so mutation of the agr system also resulted in increased ssl expression [129, 134].
Chapter 1 – Introduction

1.4.3.2. SSL immune-modulating cluster

SSLs 7-10 belong to a clade involved in modulating the immune response by binding molecules such as complement and immunoglobulins. SSL7 was the first SSL to be discovered and is the most characterised SSL. SSL7 was initially shown to induce cultured cells to produce IL-1β, IL-6 and TNF-α [114]. Subsequently, SSL7 has been identified to bind both IgA and C5 with high affinity, inhibiting the recognition by FcαRI and C5 convertase respectively [135, 136]. SSL7 binds both monomeric and dimeric IgA and prevents IgA opsonisation and subsequent cellular responses such as oxidative burst [137, 138]. SSL7 binding to C5 prevents its cleavage to C5b preventing the formation of the MAC complex on S. aureus, Lactococcus lactis and S. pyogenes [18]. More importantly, SSL7 binding to C5 prevents the release of the anaphylotoxin C5a, and has been shown to prevent the C5a-dependent activation, chemotaxis and phagocytosis of neutrophils [18, 137, 139]. A crystal structure revealed that SSL7 is able to bind both molecules simultaneously as C5 binding is via a site in the β-grasp domain while IgA binding is on the opposite side, through the OB-fold [140]. Steric interference is achieved through the formation of a pentameric structure containing two C5 molecules, two SSL7 molecules and one IgA molecule [140]. SSL7 displays species specificity in its binding of IgA and C5 [135]. The importance of SSL7 to the survival of the bacterium has been demonstrated in vivo as SSL7 diminished the influx of neutrophils in response to peritoneal immune complexes or S. aureus [18, 137]. The protective effect was heavily dependent on the ability of SSL7 to bind C5 as a mutation in the C5 binding site restored the influx of neutrophils to control levels [18].

SSL10 has been shown to specifically target primate IgG class 1 only [141, 142]. The IgG1 binding site on SSL10 has been mapped to a region close to the C1q and FcγR binding sites [142]. SSL10 has therefore been shown to inhibit the classical complement pathway and the IgG1-dependent phagocytosis of opsonised bacteria [141, 142]. SSL10 has also exhibited the ability to bind fibrinogen, prothrombin, fibronectin and plasminogen and so has an unknown activity in the coagulation system [143]. SSL10 has also been shown to bind the chemokine receptor, CXCR4, preventing the signalling and subsequent migration of tumour cells to CXCL12 in vitro [144]. There is also evidence that SSL10 binds, in a calcium-independent manner, to phosphatidylserine (PS) which is exposed in apoptotic neutrophils [145].

25
SSL9 has also been shown to inhibit complement activation in the classical and lectin pathways [146]. SSL9 directly binds the C4b2a convertase preventing the cleavage of C3 to C3b and so inhibits the opsonisation and C3b-dependent phagocytosis of *S. aureus* [146]. SSL9 has also demonstrated the ability to bind both fibrinogen and fibronectin simultaneously which is postulated to be a mechanism to concentrate SSL9 at the site of infection [146].

The last member, SSL8 has been shown to bind the extracellular matrix protein, tenascin C through its fibronectin type III repeats 1-5 (FNIII1-5) [147]. The FNIII1-5 region of tenascin C is responsible for binding fibronectin, integrins and heparin [147]. SSL8 has been shown to inhibit the interaction between TNIII1-5 and fibronectin but not integrin α9 [147]. SSL8 inhibiting the interaction between tenascin C and fibronectin delays the migration of keratinocytes along fibronectin in a scratch wound model [147].

### 1.4.3.3. SSLs in the immune evasion cluster

As mentioned previously, SSL12, 13 and 14 are located in the IEC downstream of the main cluster of SSLs. While little is known about their function, they share similar activities to the immune-modulating SSLs. SSL12, 13 and 14 have all been shown to inhibit the classical and lectin complement pathways [148]. SSL14 has been shown to directly bind C1q, which is likely to interfere with the activation of the classical pathway [148]. Like SSL9 and SSL10, SSL12, 13 and 14 are able to bind fibrinogen and fibronectin and so may interfere with coagulation [148]. SSL14 has also demonstrated the ability to bind human umbilical vein endothelial cells (HUVEC) and peripheral blood mononuclear cells (PBMC) [148].

### 1.4.3.4. Carbohydrate binding SSLs

The final major clade within the SSL family includes SSL2, 3, 4, 5, 6 and 11 (fig. 1.7). A majority of this family have been shown to bind sialylated carbohydrates through key conserved residues [117-119, 146]. Sialylated carbohydrate binding was first discovered when examining the binding properties of SSL11 whereby it was subsequently determined that SSL11 bound the sialylated carbohydrate sLe\(^x\) (discussed later) [118]. Crystal structures of SSL4, 5 and 11 have all shown a similar binding site for sLe\(^x\) (fig. 1.8) [117-119]. A 17 amino acid V-shaped depression is formed between the β10 strand of the β-grasp domain and a distorted 3\(_{10}\) α-helix [117-119].
Figure 1.8. Crystal structures of SSL4, 5 and 11 binding sLe\(^x\)

Crystal structures of SSL4, 5 and 11 (ribbons) with bound sLe\(^x\) (stick) in the V-shaped depression. The secondary structures are annotated. The binding site and orientation of sLe\(^x\) is almost identical between the three SSLs. Adapted from references [117-119].

Residues within this depression are responsible for the binding of the SSLs to sLe\(^x\). In SSL11 seven residues directly hydrogen bond to sLe\(^x\) [118]. These residues are Phe166, Thr168, Glu170, Gln176, His178, Arg179, Asp182 [118]. SSL5 has identical equivalent residues while the other members of the carbohydrate binding clade have almost identical residues (fig. 1.9). Recognising sLe\(^x\) by the SSLs involves the docking of the carboxyl end of Sia in a patch of positively charged residues (Arg158 and Arg179 for SSL11) [117-119]. Both Thr168 and Arg179 each form two hydrogen bonds with Sia and mutation of either residue abolishes the ability of the SSL to bind sLe\(^x\) [118, 119]. It is these two residues that are highly conserved and define the carbohydrate-binding clade of SSLs [123]. SSL1 contains these two residues, but has no identity with the remaining five residues and currently has not demonstrated the ability to bind carbohydrates (fig. 1.9).
Figure 1.9. Alignment of SSL proteins belonging to the carbohydrate binding clade

An alignment of SSL proteins from strain MW2, belonging to the carbohydrate binding clade was generated using Clustal Omega [149]. Only the final sequences of the β-grasp C-terminal domains are shown to highlight the carbohydrate binding residues. The highly conserved Thr168 and Arg179 are highlighted in green. Glu170, Gln176 and Asp182 are conserved among the known carbohydrate binding SSLs (yellow). The remaining two residues (blue) are variable and have implications on the space that sLe\(^x\) can enter.

SSL11 is able to make ten hydrogen bonds to sLe\(^x\) with six attaching to Sia, two to galactose and one each to fucose and N-acetylglucosamine [118]. SSL4 has a higher affinity for sLe\(^x\), when compared to SSL11, as SSL4 contains an asparagine (Asn181) at a position where SSL11 has a histidine (His178) [119]. The histidine in SSL11 angles into the pocket pushing the GlcNAc further out [119]. This is not the case in SSL4 which allows SSL4 to make four extra hydrogen bonds to GlcNAc and fucose [119]. SSL5 also contains a histidine (His185), but in a different orientation allowing for GlcNAc to occupy the same space as it does for SSL4 [119].

The function of the carbohydrate binding SSLs is less well understood. SSL3 has demonstrated the ability to inhibit TLR2 signalling by binding the TLR2 extracellular domain [150, 151]. TLR2 is important in innate immunity as it recognises pathogen associated molecular patterns (PAMPS) from a variety of microbes [152]. Of importance to Gram positive bacteria is the TLR2/TLR1 heterodimer that recognises lipoteichoic acid and the TLR2/TLR6 heterodimer that recognises diacylated lipopeptides [152]. The binding of SSL9 to TLR2 resulted in reduced IL-8, IL-12 and TNF\(\alpha\) cytokine production [150, 151]. This is unique to SSL3 as SSL11 did not exhibit similar activities [150]. SSL4 (the closest relative of SSL3) displayed significantly weaker activity when compared to SSL3 [119, 150]. SSL3 has also been shown to bind and internalise into neutrophils as well as cause neutrophil aggregation [146]. SSL3 also binds PSGL-1 and inhibits the rolling of neutrophils over P-selectin [146].
Chapter 1 – Introduction

SSL5 binds the FcαRI as well as PSGL-1, blocking their respective association with IgA and P-selectin [117, 153]. SSL5 blocking PSGL-1 binding P-selectin has been shown to not only prevent neutrophil rolling but also adhesion of leukaemia cells to endothelial cells and platelets [153, 154]. SSL5 has also been shown to bind the platelet receptor GPVI which subsequently activates platelet aggregation in a GPIbα-dependent manner [155, 156]. SSL5 has also been shown to bind pro-matrix metalloprotease-9 (pro-MMP-9) and MMP-9, preventing its activity in hydrolysing gelatin and attenuating the transmigration of neutrophils across a basement membrane [157]. Unlike SSL11, SSL5 was shown to bind and inhibit the signalling of C3a, C5a, CXCL1, CXCL8, CXCL12, CCL3, CCL5 and CX3CL1 through their target receptors [158]. This antagonism interfered with the subsequent calcium mobilisation, actin polymerisation and chemotaxis of the cells [158].

The ssl11 gene is highly conserved as it is found in most S. aureus strains [118, 120]. SSL11 was found to bind neutrophils and monocytes while a small amount of SSL11 was also bound to T cells but no binding was observed on B cells [118]. Following binding to neutrophils, SSL11 is rapidly internalised into the cells in an active process (fig. 1.10) [118].

---

**Figure 1.10. SSL11 internalisation into neutrophils**

Live cell imaging of labelled SSL11 (red) rapidly internalises into neutrophils. This process is active and is inhibited at 4°C whereby metabolic processes are diminished. From reference [118].
Chapter 1 – Introduction

Binding of SSL11 to neutrophils also results in the potent aggregation of the neutrophils [122]. SSL11 has also been shown to bind platelets most likely via direct binding to GpIIb/IIIa in a carbohydrate-dependent manner [122, 159].

SSL11 has demonstrated binding to a soluble FcαRI, but only when the source of the recombinant FcαRI was from Chinese hamster ovary (CHO) and not insect cells [118]. Based on knowledge of Drosophila recombinant glycoprotein expression, it was hypothesized that the binding was due to the presence of a carbohydrate [118]. Neuraminidase treatment of FcαRI and cells indicated that SSL11 was binding sialylated carbohydrates as it abolished binding [118]. A glycomics array revealed that SSL11 favoured binding to sialyllactosamine (sLacNAc) which contains Siaα2-3 linked to Gal (Neu5Aca2-3Galβ1-4GlcNAc) [118]. With sLacNAc being the major component of sLe^x, SSL11 was shown to be able to bind sLe^x with an affinity of 0.72 ± 0.11μM [118]. SSL11 is able to bind sLe^x exposed on the surface of PSGL-1, inhibiting its interaction with P-selectin [118]. SSL11 binding PSGL-1 suppresses neutrophil rolling by blocking the interaction with P-selectin [118, 122].

Crystal packing in developing the structure of SSL11 revealed that it is able to form dimers through hydrogen bonds between primary amines and carboxyl groups from adjacent β7-strands (fig. 1.11a) [118, 122]. Due to the curvature of the β7-strand, the dimer form a saddle-shaped groove whereby the sLe^x binding sites are both in the same plane (fig. 1.11b) [118].
1.5. Aims

Knowledge of the pathogenesis of *S. aureus* will be crucial in developing strategies to treat or even prevent infections. Evasion of the host immune system is a critical component of *S. aureus* pathogenesis as demonstrated by the vast array of virulence factors it produces. The SSLs are an important component of the immune evasive strategy that *S. aureus* employs. The importance and abundance of sialylated glycoreceptors in immune recognition makes them an obvious target for bacteria to perturb in order to evade immune surveillance. The aim of this project is to further characterise the binding, internalisation and function that SSL11 has on myeloid cells with the desire to fully understand the benefit it provides to the bacteria.
Chapter 2. Materials and Methods

2.1. Materials

2.1.1. Bacterial Isolates

2.1.1.1. Plasmids

See appendix 7.2 for plasmid details.

- pET32a-3C (pET) pET32a expression vector from Novagen with a modified 3C protease site insertion 3’ of the thioredoxin gene, trxA. This vector has an ampicillin resistance gene.

2.1.1.2. Bacterial strains

- *Escherichia coli* AD494(DE3):pLysS were purchased from Novagen. AD494 contains both chloramphenicol and kanamycin resistance genes. Contains pET32a.3C including SSL11 genes (see table 2.1).

- *S. aureus* Newman Δspa Produced by Dr. S. Holtfreter and Prof. B. Bröker, Ernst Moritz Arndt University, Greifswald, Germany. Deleted Protein A. Contains erythromycin resistance gene.

- *S. aureus* Newman GFP Obtained from Dr S. Swift, University of Auckland. Contains a plasmid expressing GFP and erythromycin resistance gene.

Table 2.1. Details on ssl11 genes cloned into pET32a.3C

<table>
<thead>
<tr>
<th>ssl11 from <em>S. aureus</em> stain</th>
<th>Strain isolated from</th>
<th>ssl11 cloned by</th>
</tr>
</thead>
<tbody>
<tr>
<td>US6610</td>
<td>Clinical isolate – Auckland Hospital, NZ</td>
<td>Dr M. Chung</td>
</tr>
<tr>
<td>US6610 R179A</td>
<td>Same as for US6610 with a point mutation</td>
<td>Mr R. Sequeira</td>
</tr>
<tr>
<td>GL10</td>
<td>Clinical isolate – Greenlane Hospital, NZ</td>
<td>Dr R. Langley</td>
</tr>
<tr>
<td>Newman</td>
<td>Gift from Dr E. Skaar, USA</td>
<td>Ms F. Clow</td>
</tr>
<tr>
<td>JSNZ</td>
<td>Preputial abscesses of C57BL/6 mice, NZ</td>
<td>Ms F. Clow</td>
</tr>
</tbody>
</table>
2.1.2. Protein Expression and Functional Analysis

2.1.2.1. Antibodies

Table 2.2. Summary of antibodies used

<table>
<thead>
<tr>
<th>Target</th>
<th>Species</th>
<th>Clonality</th>
<th>Dilution</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human vimentin</td>
<td>Mouse</td>
<td>Mono</td>
<td>1 µg/mL or 1:2000</td>
<td>Targets intermediate filaments</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-human β-actin</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1:2000</td>
<td>Targets microfilaments</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-human α-tubulin</td>
<td>Mouse</td>
<td>Mono</td>
<td>1 µg/mL</td>
<td>Targets macrofilaments</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-human giantin</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1 µg/mL</td>
<td>Golgi complex marker</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-human mannose-6-phosphate receptor</td>
<td>Mouse</td>
<td>Mono</td>
<td>1 µg/mL</td>
<td>Late endosome marker</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-human calnexin</td>
<td>Rabbit</td>
<td>Poly</td>
<td>5 µg/mL</td>
<td>ER marker</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-human protein disulfide isomerase</td>
<td>Mouse</td>
<td>Mono</td>
<td>10 µg/mL</td>
<td>ER marker</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-lysosomal associated membrane protein 1</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1 µg/mL</td>
<td>Lysosome marker</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-SSL11</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1:2000</td>
<td>Targets all SSL11 alleles</td>
<td>Dr. M. Chung</td>
</tr>
<tr>
<td>Anti-SSL11</td>
<td>Mouse</td>
<td>Mono</td>
<td>1:2000</td>
<td>Targets SSL11 US6610 only</td>
<td>See methods</td>
</tr>
<tr>
<td>Anti-Sheep RBC</td>
<td>Rabbit</td>
<td>Poly</td>
<td>1:2500</td>
<td>Opsonises RBC</td>
<td>Virion/Serion</td>
</tr>
<tr>
<td>Anti-human C5b-9</td>
<td>Mouse</td>
<td>Mono</td>
<td>1:4000</td>
<td>Detects MAC</td>
<td>Abcam</td>
</tr>
<tr>
<td>Alexa Fluor 488 label</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-rabbit IgG</td>
<td>Donkey</td>
<td>Poly</td>
<td>1:500</td>
<td>Secondary Antibody</td>
<td>Abcam</td>
</tr>
<tr>
<td>Alexa Fluor 594 label</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-mouse IgG</td>
<td>Goat</td>
<td>Poly</td>
<td>10 µg/mL</td>
<td>Secondary Antibody</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Gold label</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-mouse IgG</td>
<td>Goat</td>
<td>Poly</td>
<td>1:20</td>
<td>Secondary Antibody. Gold is 6nm in size</td>
<td>Aurion</td>
</tr>
<tr>
<td>Phycoerythrin label</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-human CD11b</td>
<td>Mouse</td>
<td>Mono</td>
<td>1:20</td>
<td>Targets Mac-1 integrin</td>
<td>BD Biosciences</td>
</tr>
</tbody>
</table>
2.1.2.2. Common Buffers and Solutions

- **2.5% Blocking Solution**: 2.5% w/v non-fat dairy milk powder in TBS
- **5% Blocking Solution**: 5% w/v non-fat dairy milk powder in TBS
- **10% APS**: 10% w/v APS in water
- **ACD**: 0.052M citric acid, 0.08M trisodium citrate, 0.18M glucose
- **Blood Buffer**: 150mM NaCl, 5mM KCl, 1mM CaCl$_2$, 1mM MgCl$_2$, 20mM HEPES pH 7.4, 10mM glucose. Filter Sterilised
- **Cell lysis buffer**: 50mM Tris-HCl pH 7.4, 300mM NaCl, 1% Triton X-100 and a tablet per 10mL of EDTA-free protease inhibitor cocktail (Roche)
- **Coomassie Blue stain**: 50% v/v ethanol, 7.5% v/v glacial acetic acid, 0.05% w/v Coomassie Brilliant Blue R250
- **ELISA Coating**: 15mM Na$_2$CO$_3$, 35mM NaHCO$_3$ pH 9.6
- **ELISA Developing**: 50mM citric acid, 100mM Na$_2$HPO$_4$ pH 9.2. To 12mL add 0.012% H$_2$O$_2$ and 1 O-phenylenediamine tablet (Life Technologies)
- **GHB** (5×): 0.75 M NaCl, 0.5% w/v bovine skin type B gelatin (Sigma Aldrich), 2mM MgCl$_2$, 0.3mM CaCl$_2$, 0.3M HEPES pH 7.35
- **GHB (5×)**: 0.75 M NaCl, 0.5% w/v bovine skin type B gelatin (Sigma Aldrich), 10mM MgCl$_2$, 10mM EGTA, 0.3M HEPES pH 7.35
- **HBS-E**: 10mM HEPES-HCl pH 7.4, 175mM NaCl, 3.4mM EDTA
- **HBSS**: Hanks balanced salt solution (Sigma Aldrich)
- **2× Loading buffer**: 100mM Tris-HCl pH 6.8, 4.0% w/v SDS, 0.2% w/v bromophenol blue, 20% w/v glycerol
- **Laemmli buffer**: 25mM Tris-HCl pH 8.8, 192mM glycine, 0.1% w/v SDS
- **NTA buffer I**: 50mM NaPO$_4$ pH 8.0, 300mM NaCl, 10mM imidazole, 10% v/v glycerol
- **NTA buffer II**: 50mM NaPO$_4$ pH 8.0, 300mM NaCl, 100mM imidazole
- **NTA buffer III**: 50mM NaPO$_4$ pH 8.0, 300mM NaCl
- **PBS**: 150mM NaCl, 2.7mM KCl, 10mM Na$_2$HPO$_4$, 1.8mM KH$_2$PO$_4$ pH 7.4 or 8.0
- **PBS-BSA**: PBS with 0.5-1% w/v bovine serum albumin fraction V (Life Technologies)
Chapter 2 – Materials and Methods

PBS-T

PBS with 0.05% v/v Tween 20

Platelet wash buffer

137mM NaCl, 2.68mM KCl, 10mM HEPES, 1.7mM MgCl₂,
25mM Glucose, 0.05% w/v human serum albumin fraction 5
pH 6.6

Ponceau S stain

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

RBC lysis solution I

0.2% w/v NaCl

RBC lysis solution II

1.6% w/v NaCl

Relaxation buffer (10x)

1M KCl, 30mM NaCl, 175mM MgCl₂, 12.5mM EGTA,
100mM PIPES pH 7.2

SDS-PAGE Solution A

30% acrylamide

SDS-PAGE Solution B

1.5M Tris pH 8.8, 0.4% w/v SDS

SDS-PAGE Solution C

0.5M Tris pH 6.8, 0.4% w/v SDS

Stripping Buffer

100mM β-mercaptoethanol, 2% w/v SDS, 62.5mM Tris.HCl
pH 6.7

TBS

10mM Tris-HCl pH 8.0, 120mM NaCl

TBS-T

TBS with 0.1% v/v Tween 20

TSA I

10mM Tris-HCl pH 8.0, 400mM NaCl, 0.1% v/v Triton X-100

TSA II

10mM Tris-HCl pH 8.0, 150mM NaCl, 0.05% w/v NaN₃

Towbin’s buffer

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

2.1.2.3. Media

Luria Bertani broth

1% w/v Bacto tryptone (Oxoid), 0.5% w/v Bacto yeast extract
(Oxoid), 1% w/v NaCl

Agar

Media broth containing 1.5% Bacto agar
2.1.3. Cell culture

2.1.3.1. Media

RPMI 1640 | Roswell Park Memorial Institute media 1640 pH 7.4 (Life Technologies) supplemented with 2mg/mL sodium bicarbonate
---|---
Complete RPMI | RPMI 1640 supplemented with 50U/mL penicillin, 50µg/mL streptomycin, 2mM L-glutamine, 110µg/mL sodium pyruvate and 10% fetal calf serum (FCS) that had been heat inactivated for 30 min at 56°C (all from Life Technologies)
THP-1 Media | Complete RPMI supplemented with 0.05mM β-mercaptoethanol (Sigma)

2.1.3.2. Cell lines

THP-1 | Monocyte cell line isolated from 1 year old patient with acute monocytic leukemia (ATCC TIB-202).

2.2. Methods

2.2.1. Protein production

2.2.1.1. Inducing protein expression for purification in E. coli

_E. coli_ containing _ssl11_ cloned into the expression vector (pET32a.3C) were inoculated into 100mL LB containing antibiotics (50µg/mL ampicillin, 34µg/mL chloramphenicol and 15µg/mL kanamycin). These were left O/N at 37°C whilst shaking. The following day, the 100mL culture was added to 900mL LB containing the same antibiotics and left shaking at 37°C until a 600nm optical density of 0.6-0.8 was achieved. The culture was then cooled to approximately 30°C before inducing expression by adding 0.1mM IPTG. This was left shaking for 4 h at 28-30°C. The bacteria were pelleted at 5000g for 15 min at 4°C and the supernatant discarded. The pellet was resuspended in NTA I and then supplemented with 0.1mM PMSF and 1% v/v Triton X-100. The suspension was stored at -80°C.
Chapter 2 – Materials and Methods

2.2.1.2. Nickel Affinity chromatography

The bacterial suspension from 2.2.1.1 was thawed and lysed using a Misonix XL2015 sonicator set to full power with a 75% pulse. 3× 1 min bursts were sufficient to lyse the cells. Bacterial debris was removed by centrifugation at 16,000g for 15 min. The soluble protein containing lysate was passed over an IDA sepharose column that had been recharged with 100mM NiSO$_4$ and equilibrated with 10cv NTA I. After passing and collecting all the lysate run-off, the column was washed with 10cv NTA I which was also collected. Finally the protein was eluted off and collected using 5cv NTA II. All the collected fractions were run on a SDS-PAGE gel to determine the approximate concentration. 10mM DTT and 3C protease (10μg per approximate mg of fusion protein) was then added to the SSL11 fusion protein containing fraction. This was left O/N at 4°C before dialysing the SSL11 fusion proteins into NTA III (for Newman and GL10 alleles) or 20mM Tris-HCl pH 8.0 (for US6610 and JSNZ alleles). The SSL11 alleles dialysed into NTA III were passed over IDA sepharose again to remove excess thioredoxin, with the unbound flowthrough containing SSL11 then being dialysed to 20mM Tris-HCl pH 8.0. SSL11 was then passed over anion exchange chromatography (section 2.2.1.3) to maximise purity of the SSL11.

2.2.1.3. Anion exchange chromatography

Recombinant SSL11 was loaded onto a MonoQ 5/50 GL (GE Healthcare) anion exchange column connected to an ÄKTA FPLC system (Amersham) that had been equilibrated with 5cv 20mM Tris-HCl pH 8.0. Typically, protein was concentrated in a 10kD Vivaspin concentrator (GE Healthcare) before loading. SSL11$_{US6610}$ and SSL11$_{JSNZ}$ were separated from thioredoxin by negative selection. These alleles don’t bind the column and are found in the flow-through. SSL11$_{Newman}$ and SSL11$_{GL10}$ bound the column and were eluted with a salt gradient reaching a maximum concentration of 1M NaCl. SSL11 was then dialysed to PBS pH 8.0.

2.2.1.4. Size exclusion chromatography

When required, SSL11 was dialysed to either PBS pH 8.0 or HBS-E. No more than 500μL of SSL11 was added to Sephadex 75 10/300 (GE Healthcare) pre-equilibrated in appropriate buffer. SSL11 was run through at 1mL/min on the ÄKTA FPLC system (Amersham) and the dimer and monomer size separated peaks were collected.
2.2.1.5. Production of monoclonal anti-SSL11

SSL11\_US6610 was used as an inoculum for the generation of monoclonal antibodies directed against SSL11. Handling of the animals was performed by Dr F. Radcliff in the Vernon Jansen Unit, University of Auckland. The generation of stable SSL11 producing hybridomas was performed by Ms R. Yugova and Dr. F. Radcliff. Hybridomas were grown in 20mL RPMI 1640 with 5% FCS at 37°C 5% CO\_2 and maintained below 1×10\(^6\)cells/mL. Production of anti-SSL11 antibodies was confirmed by ELISA. Growth was up-scaled to 900mL and the cells left for two weeks at 37°C with 5% CO\_2. The hybridomas were removed by centrifugation at 400g. The supernatant was filtered before being passed over a Protein G sepharose column (GE Healthcare) equilibrated with 20mM Tris-HCl pH 7 using an ÄKTA FPLC system (Amercham). Bound monoclonal antibody was eluted with 100mM glycine pH 3 before being dialysed and stored in PBS pH 8.0.

2.2.2. Protein Analysis

2.2.2.1. Making acrylamide gels for SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were made to contain a desired percentage of acrylamide to allow for optimal separation. Typically 12.5% was used with 17% being for smaller proteins and 10% for larger proteins. The running gel solutions (table 2.3) were applied to assembled plates, followed by the application of a small volume of water-saturated butanol to level off the interface. This was allowed to polymerise before draining off the butanol. Stacker solution (table 2.3) was then applied to the top of the running gel and a comb applied. This again was allowed to polymerise and the gel was then used immediately or stored in moist tissue at 4°C.

<table>
<thead>
<tr>
<th>Table 2.3. Making acrylamide gels for SDS-PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Solution A</td>
</tr>
<tr>
<td>Solution B</td>
</tr>
<tr>
<td>Solution C</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
<tr>
<td>10% APS</td>
</tr>
</tbody>
</table>
2.2.2.2. Protein separation by SDS-PAGE

Protein samples were mixed with an equal volume of 2× loading buffer for a non-reducing gel. β-mercaptoethanol at 300mM was then added to the 2× loading buffer to obtain a reducing gel. Samples were then denatured at 94°C for 2 mins followed by a brief centrifuge. The samples were then added to an appropriate gel and electrophoresed at 200V with a current of 20mA per gel in Laemmli buffer using Mighty Small II (GE Healthcare). All gels used were 12.5% unless otherwise stated.

2.2.2.3. Staining of SDS-PAGE separated proteins with Coomassie blue stain

Following the completion of SDS-PAGE, the gels were left to stain in Coomassie blue for at least 15 min with gentle shaking (~50rpm). The gel was then rinsed with deionised water before being destained with 25% v/v ethanol and 8% v/v glacial acetic acid. Tissue was applied to absorb excess dye.

2.2.2.4. Staining of SDS-PAGE separated proteins with silver nitrate

Silver staining provides greater sensitivity for detecting proteins of Coomassie stained gels. Following destain, the gels are washed in deionised water for several minutes before being incubated in 0.02% Na2S2O3 for 5 min. The gel was then rinsed briefly with water before being incubated with 0.1% w/v AgNO3 for 10 min. After another rinse, the stain was developed with 3% Na2CO3, 0.05% formaldehyde and 0.0004% Na2S2O3 until bands appeared and turned brown. Then 2.3M acetic acid was added to stop the reaction.

2.2.2.5. Identification of SDS-PAGE separated proteins by Mass Spectrometry

Bands of interest were excised from Coomassie stained gels that had not been exposed to tissue absorbants, and diced into small cubes. These were sent to the Proteomics Facility, University of Auckland for processing and identification using the LTQ-FT mass spectrometer (Thermo Scientific). Data was analysed online using MASCOT MS/MS ion search (www.matrixscience.com). Similarly, whole cell lysates from 2.2.7.3 were processed and analysed at the Applied Proteomics Group, Ernst Moritz Arndt University, Greifswald using a Q Exactive LC/MS (Thermo Scientific).
2.2.3. Protein Modifications

2.2.3.1. Coupling SSL11 to cyanogen bromide (CnBr) activated sepharose

CnBr activated sepharose (GE Healthcare) was swelled with 1mM HCl before washing the sepharose slurry through a sintered glass filter with 1mM HCl. The sepharose was transferred to a falcon tube (BD Biosciences) and mixed with an equal volume of 1mM HCl. The sepharose was allowed to settle and excess supernatant was removed and equilibrated twice with PBS pH 8.0. Protein at 2mg/mL in PBS pH 8.0 was added to 600µL of the washed sepharose. Proteins were incubated O/N at 4°C with constant mixing by inversion. The supernatant was removed and examined by UV spectroscopy at 280nm to monitor the success of coupling. Exposed areas on the sepharose were blocked with two washes with 100mM Tris-HCl pH 8.0 and 150mM NaCl and then incubated O/N at 4°C. The supernatant was removed and the beads washed six times with PBS pH 8.0. The protein-coupled sepharose was resuspended in an equal volume of PBS pH 8.0 containing 0.025% w/v sodium azide and stored at 4°C.

2.2.3.2. Fluorescein labelling of proteins

All reactions were performed in the dark to minimise photobleaching. Succinimidyl ester 5-(6)-carboxyfluorescein diacetate (Life Technologies) was dissolved at 4mg/mL in dimethyl sulfoxide (DMSO) and used immediately. The FITC solution was added dropwise to 2mg/mL protein dissolved in PBS or NaPO₄ pH 8.0. Once the solution became sufficiently yellow (generally 10µL), the reaction was incubated O/N at 4°C with continual mixing by inversion. Free FITC was removed by gel filtration through a 10mL G-25 sephadex (GE Healthcare) column using gravity feed. The efficacy of coupling was determined using UV spectroscopy where a 280nm and 495nm absorbance ratio of between 0.3 and 0.7 is optimal.

2.2.3.3. Biotinylation of SSL11

SSL11 at 2mg/mL in PBS pH8.0 was biotinylated with 10mM EZ-Link sulfo-NHS biotin (Pierce) for 1 h at RT with gentle rotation. Excess biotin was removed by extensive dialysis in PBS pH 8.0.
2.2.3.4. Attachment of a transferable linker to SSL11

A 2µL volume of 0.05mg/µL Sulfo-SBED (Thermo) dissolved in DMSO was added to 750µL SSL11. This was incubated at 4°C for 2 h in the dark with gentle rotation. Excess linker was removed by extensive dialysis into PBS pH 8.0 before being centrifuged and filtered to remove any precipitate.

2.2.3.5. Alexa Fluor labelling of SSL11

Alexa Fluor 488 or 647 (Life Technologies) was resuspended in DMSO to make a stock of 10mg/mL. A 10µL volume of the dye was added to 500µL of SSL11 at 2mg/mL in PBS pH 8.0 and 100mM NaHCO₃. The solution was left rotating O/N at 4°C in the dark. Free dye was removed by passing the mixture over a G-25 sephadex column and extensive dialysis into PBS pH 8.0. The degree of labelling and protein concentration was calculated as per the manufacturer’s instructions.

2.2.4. Cell Culture

2.2.4.1. Isolation of plasma, mononuclear cells and granulocytes

Fresh blood was collected from healthy human volunteers in a liquid K₃EDTA or Heparin vacutainer tube (BD Biosciences). The cells were isolated using density gradient centrifugation, achieved by layering 2.5mL Histopaque 1077 (Sigma Aldrich), in a 15mL falcon tube (BD biosciences), onto a layer of 2.5mL Histopaque 1119 (Sigma Aldrich). A 4-5mL volume of the collected blood was carefully layered onto the Histopaque gradient before centrifugation at 700g for 30 min with no brake. The resulting plasma, mononuclear cell and granulocyte layers were transferred to individual 50mL falcon tubes. The plasma was kept on ice for future use. The mononuclear cells (monocytes, lymphocytes and platelets) and granulocytes (mostly neutrophils with some eosinophils) were washed in 50mL filtered PBS and centrifuged at 300g for 5 min with a low brake. The granulocytes were often contaminated with erythrocytes, so the erythrocytes were lysed by adding 15mL 0.2% w/v NaCl for 30 sec before adding 15mL 1.6% w/v NaCl. The cells were pelleted by centrifugation at 300g for 5 min with a low brake. The PBS wash step as above was repeated twice. The quantity of cells was determined by light microscopy using a haemocytometer with trypan blue to give contrast and indicate cell viability. Monocytes were further isolated from PBMCs by adhering 2×10⁶ cells/mL in RPMI 1640 to plastic tissue culture dishes (BD
Chapter 2 – Materials and Methods

Biosciences) for 1 h [160]. Non-adhered cells were removed by washing. All steps were performed at RT.

2.2.4.2. Isolation of platelet rich or poor plasma

Healthy fresh blood was collected from donors that had not taken aspirin in the last 2 weeks. The blood was drawn into 0.105M sodium citrate tubes (BD Biosciences) and centrifuged at 200g for 30 min with no brake. The top ¾ of the plasma layer was transferred to a 15mL falcon tube (BD Biosciences) with care not to take leukocytes and erythrocytes that had aggregated at the bottom. This plasma is platelet rich and is known as platelet rich plasma (PRP). The PRP was used as is or it was centrifuged at 1000g for 15 min with a low brake. The supernatant was removed and discarded or kept as platelet poor plasma (PPP).

2.2.4.3. Isolation of serum

Healthy fresh blood was collected in serum vacutainer tubes (BD Biosciences). The blood was left to stand at RT for 15 min to allow adequate clotting. The blood was then centrifuged at 1250g for 20 min and the upper serum layer was collected. If using in conjunction with leukocytes, the serum would be from the same donor.

2.2.4.4. THP-1 cell culture

THP-1 cells were initially inoculated into 10mL THP-1 medium and grown at 37°C 5% CO₂. The density was maintained below 1×10⁶ cells/mL. This was scaled-up to acquire the desired amount of cells when required.

Differentiation of THP-1 monocytes to macrophages was achieved as recommended by Daigneault et al. [161]. This was achieved by incubating 2×10⁵ THP-1 cells in THP-1 medium with 200nM phorbol-12-myristate-13-acetate (PMA) (Sigma Aldrich) for 3 days at 37°C 5% CO₂. The cells were then allowed to recover for 5 days in THP1 medium at 37°C 5% CO₂ before use.
2.2.4.5. Differentiation of primary human monocytes to macrophages

Healthy macrophages were differentiated from human monocytes as described by Daigneault et al. with minor modification [161]. Mononuclear cells isolated from 2.2.4.1 were resuspended at $2 \times 10^6$ cells/mL in RPMI 1640 containing 10% serum (from the same donor – see 2.2.4.3). A 1mL volume of the cell suspension was added to a 3mL L-lysine coated glass bottom dishes (World Precision Instruments) and incubated O/N at 37°C 5% CO₂. Alternatively mononuclear cells were resuspended at $6 \times 10^6$ cells/mL in RPMI 1640 containing 10% serum with 1mL being added to 6 well tissue culture plates (BD Biosciences) and incubated O/N at 37°C 5% CO₂. The medium was removed and the adherent cells were maintained for 14 days in complete RPMI with regular media changes.

2.2.4.6. Bacterial growth

*S. aureus* Newman Δspa and *S. aureus* Newman GFP were grown in LB containing 30µg/mL erythromycin O/N at 37°C with shaking.

2.2.4.7. Heat killing of *S. aureus*

Strains grown from 2.2.4.6 were washed 3 times with sterile PBS pH 7.4 by centrifugation at 4500g for 5 min. The bacterial pellet was resuspended in 5mL sterile PBS and incubated at 75°C for 15 min. Complete death was confirmed by growing on LB agar. The amount of bacteria were determined by optical density at 600nm.

2.2.4.8. Activation of serum complement by *S. aureus*

Heat killed *S. aureus* from 2.2.4.7 were resuspended at $2 \times 10^7$ cfu/mL in blood buffer containing 10% human serum. This was incubated at 37°C for 20 min and classified as activated serum. If desired, bacterial cells were removed by centrifugation at 4500g.
Chapter 2 – Materials and Methods

2.2.5. Analysis of Protein Interactions

2.2.5.1. Detergent lysis of purified cells

Leukocytes were lysed by adding desired volumes of cell lysis buffer to the cell pellet or monolayer. Cells were incubated for 1 h at 4°C with gentle rotation or periodic agitation. The lysed cells were then centrifuged at 16,000g at 4°C for 10 min to remove cell debris. The supernatant was then aliquoted and stored at -80°C for subsequent use.

2.2.5.2. Pulldown assay

A 10μL volume of SSL11 coupled sepharose from section 2.2.3.1 was added along with 100μL of cell lysates, to 400μL of TSA I. The solution was incubated for 1 h at 4°C with constant mixing by inversion. The beads were pelleted by centrifugation at 16,000g for 30 sec. The supernatant was removed with care as to not remove the beads. The beads were then washed 3 times with TSA I before a final wash with TSA II. The supernatant was discarded and the beads resuspended in an equal volume of 2× loading buffer. The solution was boiled at 94°C for 2 min before running on a SDS-PAGE gel with visualisation by Coomassie staining. The separated bands could then be identified by mass spectrometry.

2.2.5.3. Transfer of biotin marker to cellular components

The transferable SSL11-linker created in 2.2.3.4 was added to various cells at specified concentrations in PBS pH 7.4. SSL11 was allowed to incubate with the cells for 30 min at RT in the dark before unbound SSL11 was removed by two washes with PBS pH 7.4. Adherent cells had all their buffer removed, whereas non-adherent cells were resuspended in 20µL PBS pH 7.4. The tissue culture plate containing the adherent cells was inverted and placed on a UV transilluminator. For the non-adherent cells, the 20µL was placed onto the UV transilluminator. Similarly, 20µL of SSL11 or SSL11-linker was placed onto the UV transilluminator. All samples were exposed to UV for 10 min. The cells were then lysed as per 2.2.5.1 before being run on a SDS-PAGE gel followed by Western analysis. Detection was achieved with 1:2000 streptavidin HRP (BD Biosciences).
2.2.5.4. Western identification and analysis of proteins

Proteins run on a SDS-PAGE gel were transferred to a nitrocellulose membrane (Pall Life Sciences) using a TE77 semi-dry transfer unit (Hoefer) at 50mA/gel for 1 h. Success of transfer was analysed by examination of the Benchmark Prestained Ladder (Life Technologies) or by using a few drops of Ponceau S reversible stain which is destained by washing with deionised water. The membrane was then blocked with 5% blocking solution O/N at 4°C. The membrane was then probed with HRP conjugated to streptavidin (BD Biosciences) or primary antibody in TBS-T and incubated for 1 h at RT with shaking (50rpm). Unbound probe was removed by washing three times with TBS-T for 5 min each at RT with shaking. If additional probes were required for HRP detection, the 1 h incubation step was repeated with secondary antibody and washed as above. Immobilised complexes were visualised using chemiluminescence achieved through the Supersignal ECL Western Blotting Detection Reagents (Thermo Scientific) and detected by the Fujifilm LAS-3000 developer (Alphatech). If required, membranes were stripped by incubating the membrane in stripping buffer at 50°C for 30 min. The membrane was then extensively washed with TBS-T and the procedure repeated from the blocking step.

2.2.5.5. Biosensor analysis of SSL11 interaction with sLe\(^\alpha\) and sLacNAc

Biosensor analysis of protein interactions were performed on a Biacore T200 (GE Healthcare). BSA-sLe\(^\alpha\) (Dextra Laboratories, UK) was coupled to a CM5 chip at 100-200 RU using carbodiimide chemistry as per the manufacturer’s instructions (GE Healthcare). Similarly BSA-sLacNAc (Dextra Laboratories, UK) was coupled using the same chemistry by Mr. S. Hermans. A BSA control channel was made for the subtraction of non-specific binding. SSL11 alleles were first separated by size exclusion (see 2.2.1.4) before being passed over the Biacore T200. HBS-EP+ containing 0.05% surfactant P20 was used as the running buffer (GE Healthcare). SSL11 was passed over the chips at 20μL/min for 300 sec. The chips were regenerated with 2M MgCl\(_2\). The response sensogram plateau was taken as the response at equilibrium (R\(_{\text{eq}}\)). The K\(_D\) was determined by the Biacore T200 Evaluation software using a single-site binding model for steady state affinity. Kinetic analysis to determine association and dissociation rates was performed using the BIAevaluation software v4.1.
Chapter 2 – Materials and Methods

2.2.6. Interactions with cells

2.2.6.1. SSL11 competition for neutrophil receptors

Purified neutrophils were resuspended at $1 \times 10^7$ cells/mL in PBS pH 7.4 containing 0.5% BSA. A 100μL volume of the cells were incubated with varying concentrations of SSL11US6610. These were allowed to incubate for 30 min at RT or on ice. The cells were washed twice with centrifugation at 300g for 5 min to pellet the cells. The cells were then incubated for 30 min at RT with 10nM fluorescein-labelled SSL11US6610. The cells were again washed twice with centrifugation at 300g for 5 min before being measured by flow cytometry using a LSR II (BD Biosciences) with FACSDiva v 6.1.3 software. Subsequent analysis and gating was performed using FlowJo v10.

2.2.6.2. Neutrophil aggregation

Purified neutrophils were resuspended at $1 \times 10^7$ cells/mL in PBS pH 7.4. A 100μL volume of the cells were incubated with varying concentrations of SSL11 for 45 min in an eppendorf tube at RT. The cells were gently mixed and transferred to a 24 well tissue culture plate (BD Biosciences) and imaged using a Nikon Eclipse TE2000-S inverted light microscope. If chemical inhibitors were included, they were added at specified concentrations (see 2.2.8.6) 15 min prior to adding SSL11. Resizing of images performed using ImageJ v1.46.

2.2.6.3. Platelet aggregation and clotting

PRP or PPP isolated in 2.2.4.2 was diluted to 50% with PBS pH 7.4. SSL11US6610 was added at varying concentrations before initiating clotting with CaCl$_2$ or ADP to final concentrations of 10mM and 10µM respectively. The reaction was incubated for 1 h with constant optical density measuring at 405nm and 620nm by the μQuant spectrophotometer.
2.2.7. Functional analysis

2.2.7.1. Complement Haemolytic Assay

The CH50 total complement haemolytic assays were performed in glass tubes. Sheep erythrocytes (Life Technologies) were spun at 1250 g for 5 min at 4°C. They were then incubated in GHB++ buffer for 15 min at 37°C to lyse any unstable erythrocytes. The erythrocytes were washed in GHB++ until the background supernatant was clear. A 1 % (v/v) suspension of sheep red blood cells (sRBC) was sensitised with 1:2500 anti-sheep RBC antibodies for 30 min at 37°C. Designated concentrations of SSL11US6610 were added to 100µL sensitised sRBC before adding 100µL of 1/30 guinea pig serum (GPS). The reaction was allowed to proceed for 1 h at 37°C with periodic agitation. The experiment was exhausted by adding cold 1.2mL saline followed by pelleting of unlysed sRBC at 1250g for 5 min at 4°C. A 100µL volume of supernatant was transferred to a 96 well tissue culture plate (BD Biosciences) and the absorbance measured at 412nm.

2.2.7.2. Human complement ELISA

Heat-aggregated IgG mimics the antigen-antibody complex required to activate the classical complement pathway. Mannan activates the lectin complement pathway. Bacteria surface proteins activate the alternative pathway in the presence of EGTA which prevents classical and lectin pathways which are dependent on calcium. ELISA coating buffer containing: 25µg/mL heat-aggregated human IgG (provided by Ms. W. Hou); 20µg/mL Mannan (Sigma); or 5×10⁷cfu/mL of heat killed S. aureus Newman ΔSpA. A 100µL volume was added to Maxisorb ELISA plates (Nunc) and incubated O/N at 4°C. Plates were washed in PBS-T before being blocked with 1% Human serum albumin fraction V (Merck) in PBS pH 7.4 for 1 h at RT. To the wells containing IgG or mannan, 4% human serum in GHB++ with or without an equal volume of SSL11 was added. To the wells containing S. aureus, 10% human serum in GHB with or without an equal volume SSL11 was added. The plates were incubated at 37°C for 1 h before being washed with PBS-T. The activation of the complement pathway was identified by the addition of 1:4000 anti-human C5b-9 for 1 h at RT. This was washed with TBS-T and the secondary anti–mouse HRP was added at 1:10,000 before being developed with ELISA developing solution. Signal was measured at 490nm on a µQuant spectrophotometer. Controls included no antigen and no primary antibody.
Chapter 2 – Materials and Methods

2.2.7.3. Activation of neutrophils

Isolated neutrophils were resuspended at $5 \times 10^7$ cells/mL in PBS pH 7.4. PBS or 1µM SSL11US6610 was added to the neutrophils and incubated at RT for 45 min. A 100µL volume of the chemoattractant, N-formylmethionyl-leucyl-phenylalanine (fMLF) in DMSO was added to 100µL of neutrophils to obtain a final concentration of 10µM. This was incubated for 1-20 min at RT before being centrifuged at 400g for 2 min. Alternatively, PMA in DMSO was added at 100nM for 2 min. The neutrophil pellet was snap frozen in liquid nitrogen and stored at -80°C until required. The pellet was thawed and resuspended in 8M Urea in 20mM HEPES pH 8.0 and sonicated on ice with short bursts at the lowest power using the QSonica sonicator. The protein concentration was determined by Bradford analysis (Bio-Rad) as per the manufacturer’s instructions. Equal protein amounts were loaded and run on a SDS-PAGE gel and analysed by Western blot.

2.2.7.4. Cell death of neutrophils

Isolated neutrophils were resuspended at $1 \times 10^6$ cells in incomplete RPMI in a FACS tube (BD Biosciences). To appropriate samples, 0.25µM SSL11 was added and were left to incubate at RT for the appropriate amount of time. All samples were prepared so that the incubations would terminate at the same time. A positive control sample was prepared by adding cold absolute ethanol to neutrophils followed by a 5 min incubation at RT. The cells were washed and resuspended in 500µL incomplete RPMI containing 50µg/mL propidium iodide (Life Technologies) and counted on the LSR II flow cytometer (BD Biosciences). Counting and analysis were performed using FACSDiva v6.1.3 and FlowJo v10 respectively.

2.2.7.5. Apoptosis of neutrophils

Isolated neutrophils were resuspended at $1 \times 10^7$ cells/mL in PBS pH 7.4. A 50µL volume of neutrophils were placed into a FACS tube (BD Biosciences) and to appropriate samples 50µL of 1µM SSL11 was added and incubated at RT for 30 min. Samples were washed by centrifugation at 300g for 5 min with blood buffer, and resuspended in 100µL blood buffer. A 5µL volume of recombinant fluorescein-labelled AnnexinV (generated by Ms F. Clow) was added to the samples and left for 30 min at RT in the dark. The cells were washed and resuspended in 500µL blood buffer containing 50µg/mL propidium iodide (Life Technologies) and counted on the LSR II flow cytometer (BD Biosciences). Apoptotic neutrophils as a positive control were generated by the UV irradiation of neutrophils in
complete RPMI for 5 min before being incubated at 37°C 5% CO₂ for 2 h. Counting and analysis were performed using FACSDiva v6.1.3 and FlowJo v10 respectively.

2.2.7.6. Calcium mobilisation in neutrophils

Isolated neutrophils from heparinised blood were resuspended at 6×10⁶ cells/mL in blood buffer. To the cells, 2µM of the calcium fluorescent dye Fluo-3 (Life Technologies), was added which were then incubated at 37°C for 30 min. The cells were washed by centrifugation at 400g and resuspended in blood buffer and left for 30 min in the dark at RT to allow de-esterification of the dye. Cells were washed and resuspended in blood buffer lacking CaCl₂ with the addition of SSL11US6610 or CHIPS (generated by Dr. R. Langley) to appropriate samples for 20 min at RT. Neutrophils were washed and resuspended in blood buffer lacking CaCl₂. The samples were constantly measured by flow cytometry on the LSR II (BD Biosciences) for 5 min. After 1 min 1nM recombinant C5a (generated by Ms F. Clow) or 50µL activated serum (2.2.4.8) was added and the responses measured for the remaining 4 min.

2.2.7.7. Live cell imaging of neutrophils responding to S. aureus

Live cell imaging of neutrophils was adapted from Marks et al.[162]. L-lysine coated glass dishes (World Precision Instruments) were treated with sterile 1% gluteraldehyde in PBS pH 7.4 for 30 min and then subsequently washed with sterile PBS pH 7.4. Heat killed S. aureus GFP from 2.2.4.7 were spun at 200g for 10 min. A 1mL volume of the supernatant, which has less aggregated bacteria, were added to the gluteraldehyde treated dishes and incubated for 1 h at RT in the dark. Unbound bacteria were washed with sterile PBS pH 7.4 and free aldehyde groups were blocked with sterile 0.1% w/v L-lysine in PBS pH 7.4 for 1 h at RT in the dark. Excess L-lysine was washed away with sterile PBS pH 7.4. Human serum was diluted to 10% in sterile blood buffer and added to the fixed bacteria and left at 37°C for 10 min. Neutrophils at 1×10⁵ cells/mL in sterile blood buffer were incubated with SSL11US6610 if desired for 10 min. The neutrophils were then added to the serum opsonised S. aureus and time-lapsed imaged using the Olympus FV1000 confocal laser scanning microscope for 30-45 min with images captured every 30 sec. The final magnification for all images was 600× which were analysed using Fluoview v1.7b. Images were subsequently resized using ImageJ v1.46.
2.2.8. Analysing SSL11 in cells

2.2.8.1. Cleavage of SSL11 in neutrophils

Neutrophils at 1×10^6 cells/mL were incubated with 1µM biotin-conjugated SSL11_{US6610} in PBS for 45 min at RT. Cells were washed 3 times with PBS by centrifugation at 300g for 5 min. The neutrophil pellet was lysed as per 2.2.5.1 and run by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed with 1:1000 streptavidin HRP (BD Biosciences) and detected as mentioned in 2.2.5.4.

2.2.8.2. Subcellular fractionation of SSL11 in neutrophils

Neutrophil fractionation was performed as published [163-165]. Neutrophils at 3-5×10^6 cells/mL were incubated with 0.5-1µM biotin-conjugated SSL11_{US6610} for 30 min in PBS pH 7.4. Excess SSL11 was washed with PBS pH 7.4 by centrifugation at 300g for 5 min. The cells were resuspended in 9mL relaxation buffer and then placed in the nitrogen cavitation bomb whereby the cells were ruptured at 350-400psi for 30 min. The lysate was collected into 1.25mM EGTA and spun at 3000g for 10 min. The supernatant represents the post-nuclear supernatant (PNS) which was then layered onto a trilayer of Percoll (GE Healthcare). The Percoll trilayer was made with 9mL each of 1.12g/mL, 1.09g/mL and 1.05g/mL starting with the heaviest at the bottom. The tube was sealed and spun at 37,000g in a T-865 Sorvall rotor loaded in a Thermo Sorvall WX Ultra Series centrifuge. Fractions of 1mL were collected from the bottom of the tube and stored at −80°C until required.

2.2.8.3. Gelatin gels to test activity of gelatinase

SDS-PAGE gels with 10% acrylamide were made as for 2.2.2.1 with the addition of 0.1% w/v porcine gelatin type I (Sigma Aldrich) and without bromophenol blue. Samples from 2.2.8.2 were run on the gelatin gels without boiling and β-mercaptoethanol. The gel was then soaked in 2.5% v/v Triton X-100 for 1 h at RT on a rotary shaker. The gel was then soaked in 50mM Tris pH 7.5, 200mM NaCl, 5mM CaCl₂ and 0.02% v/v Tween 20 for greater than 18 h at 37°C. The gels were then stained for 3h at RT in Coomassie stain on a rotary shaker.
2.2.8.4. Live confocal imaging of SSL11 internalisation

Neutrophils at 1-5×10⁵ cells were allowed to adhere to L-lysine coated glass bottom dishes (World Precision Instruments) for 30 min at RT in PBS pH 7.4 while adherent macrophages had their medium washed off. SSL11 conjugated to fluorescein, Alexa Fluor 488 or Alexa Fluor 647 was added to the adhered cells and incubated for 45 min RT in PBS pH 7.4. Excess SSL11 was washed away with PBS pH 7.4 before being viewed by the Olympus FV1000 confocal scanning microscope at 600× magnification. The analysis software used was Olympus Fluoview v1.7b with resizing performed by ImageJ v1.46.

2.2.8.5. Live imaging of organelle localisation

Live organelle dyes from Life Technologies were added before SSL11 was as per 2.2.8.4. ER tracker green was added to the macrophages at 500nM in HBSS (Sigma Aldrich) containing 1mM CaCl₂ and 1mM MgCl₂ and incubated at 37°C 5% CO₂ for 30 min. Similarly tubulin tracker green was added to macrophages at 400nM and incubated at 37°C for 30 min. NBD C₆-ceramide was added to macrophages at 5µM in HBSS for 30 min at 4°C. These were washed with blood buffer and left at 37°C for 30 min in blood buffer. The macrophages were then viewed by the Olympus FV1000 confocal scanning microscope at 600× magnification. The analysis software used was Olympus Fluoview v1.7b and ImageJ v1.46.

2.2.8.6. Chemical inhibition of SSL11 entry

Neutrophils were prepared as in 2.2.8.4. However, 15 min before the addition of SSL11, chemical inhibitors were added. The chemical inhibitors include: 0.45M sucrose in PBS; 50µM chlorpromazine (Sigma Aldrich) in water; 100µM phenylarsine oxide (Sigma Aldrich) in DMSO; 10µM Pitstop (Abcam) in DMSO and 5µg/mL Filipin (Sigma Aldrich) in DMSO. The chemical inhibitors were included in all subsequent buffers. PBS and 1/1000 v/v DMSO were used as controls.

2.2.8.7. Imaging of fixed specimens

The medium was removed from macrophages before 0.25µM SSL11 Alexa Fluor 488 or 647 was added and incubated for 45 min at RT in PBS pH 7.4. Free SSL11 was removed by washing before cold methanol was added for 5 min to fix the cells. The methanol was removed and washed three times with PBS pH 7.4. The cells were permeabilised with 0.2% w/v saponin from Quillaja bark (Sigma Aldrich) for 10 min at RT and followed by three
Chapter 2 – Materials and Methods

washes with PBS pH 7.4. The cells were blocked with PBS-BSA for 1 h at RT. Desired antibody was added in PBS-BSA and left O/N at 4°C. The cells were then washed three times with PBS pH 7.4 followed by the addition of the appropriate secondary antibody in PBS-BSA for 1 h at RT. The antibody was washed away and the glass bottom cut out and mounted on a slide with Prolong Gold Antifade (Life Technologies). Controls included secondary only and monoclonal anti-SSL10 which is unreactive to SSL11.

For actin, an alternative procedure was used. Macrophages or neutrophils were fixed with 3.7% w/v formaldehyde in PBS pH 7.4 for 10 min at RT. Formaldehyde was washed away three times with PBS pH 7.4 before 20°C acetone was added for 5 min. The acetone was removed by three washes with PBS pH 7.4 and 5U phalloidin Texas red or phalloidin Alexa Fluor 488 was added in PBS pH 7.4 for 20 min at RT. SSL11 conjugated to Alexa Fluor 488 or 647 was added either prior to fixing or after the phalloidin incubation at 0.25 µM. Slides were imaged on the Olympus FV1000 confocal laser scanning microscope with a 1000× magnification. Analysis of images performed using Fluoview v1.7b and ImageJ v1.46.

2.2.8.8. Transmission Electron Microscopy

Neutrophils at 1×10⁷ cells/mL were incubated with 0.5 µM SSL11 US6610 in PBS pH 7.4 for 45 min at RT. Cells were washed two times with PBS pH 7.4 and resuspended to 1mL PBS pH 7.4. A 1mL volume of 0.1% glutaraldehyde in 0.1M NaPO₄ pH 7.2 was added to the suspension and left for 15 min at RT. The neutrophils were pelleted by centrifugation at 300g for 5 min before being submerged in 1.25% glutaraldehyde in 0.1M NaPO₄ pH 7.2 for 45 min RT. The cells were washed three times with 0.1M NaPO₄ pH 7.2. The pellet was then dehydrated by: two 5 min 70% ethanol washes at 4°C; two 5 min 80% ethanol washes at 4°C; one 10 min 90% ethanol wash at 4°C; two 10 min 100% ethanol washes at 4°C and finally one 10 min 100% ethanol wash at RT. The cells were equilibrated for 15 min at RT with a 1:1 mixture of 100% ethanol and LR White resin (ProSci Tech) followed by a 30 min incubation with a 1:2 mixture of 100% ethanol and LR White at RT. The LR White resin was then allowed to infiltrate for 1 h at RT before being replaced with fresh LR White and left rotating O/N at RT. The LR White was exchanged again and left for 1h RT before the cell pellet was transferred to an embedding mould capsule. The capsule was filled with LR White and left at 55°C for 22 h to polymerise. The embedded neutrophils were sectioned to ~80nm and mounted on EM grids by Ms H. Holloway. The grids were washed 3 times with PBS pH 7.4 before being blocked with PBS-BSA for 1 h. The grids were washed twice with PBS pH
7.4 before 25µg/mL mouse anti-SSL11 was added and incubated for 2 h at RT in PBS pH 7.4. The grids were then washed three times in 0.1% w/v BSA in PBS pH 7.4. The gold-conjugated secondary antibody was then added and left for 1 h in PBS pH 7.4. The grids were then washed three times in 0.1% w/v BSA in PBS pH 7.4 and then washed twice in PBS pH 7.4. The grids were stained with uranyl acetate for 30 min before being washed twice with MilliQ water. The grids were viewed on the Tecnai G² Spirit Twin transmission electron microscope at a 97,000× magnification.

2.2.9. Phylogenetic analysis

2.2.9.1. Protein sequence alignment

An alignment of several SSL11 protein sequences (excluding their signal peptides) was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) [149, 166]. All sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/).

2.2.9.2. Generation of SSL11 phylogenetic tree

The alignment of SSL11 protein sequences from 2.2.9.1 was used to create a Whelan and Goldman Maximum Likelihood phylogenetic tree. This tree was created using gamma distribution with invariant sites in the software Mega v5.05 [167]. The default settings of 5 discrete gamma categories was used. Missing data was completely deleted and the ML heuristic method used was Nearest-Neighbor Interchange.
Chapter 3. Analysing SSL11 interactions

3.1. Introduction

The ssl11 gene has been identified in all sequenced genomes of *S. aureus* [115, 118, 120]. SSL11 is able to bind the ubiquitous carbohydrate moiety known as trisaccharide sialyllactosamine (Neu5Aco2-3Galβ1-4GlcNAc) [117-119, 150]. SSL11 belongs to a clade along with SSLs 2 to 6 which all contain the required residues for binding the sialylated carbohydrates. The strong conservation of the SSL11 gene despite the retention of the other carbohydrate binding SSLs suggests that it has a non-redundant role. SSL11 has been shown to bind PSGL1 and the FcαR1 and following binding cells, is rapidly internalised [118]. This section aims to further characterise the interaction of SSL11 with myeloid cells with particular focus in examining if there are any differences in binding afforded by the SSL11 dimer.

3.2. Results

3.2.1. Analysis of SSL11 from sequenced *S. aureus*

With the advent of newer and cheaper sequencing platforms, more genomes of *S. aureus* have become publicly available for analysis. An alignment of several SSL11 protein sequences demonstrated that there is a large amount of variation for a majority of the protein, with the exception of the terminal β-grasp domain which is highly conserved (see appendix 7.1). The conserved residues were highlighted in the crystal structure of SSL11 (PDB file 2RDG) using PyMOL (DeLano Scientific LLC) (fig. 3.1). As is evident, strongest sequence conservation is centred around the carbohydrate binding site. There are conserved residues that wrap around the entire carbohydrate molecule which are most likely important in maintaining the structure and stability of the carbohydrate binding site and fold of the protein. This highlights the importance of the carbohydrate binding to the functionality of this protein. Using the SSL11 alignment, a Whelan and Goldman maximum likelihood phylogenetic tree was created (fig. 3.2). If the Clonal Complex (CC) has been determined, it is annotated behind the *S. aureus* title. The CC is a classification of *S. aureus* based on the presence of certain genes as determined by multi-locus sequence typing (MLST) [168].
Figure 3.1. Representation of conserved amino acids in the structure of SSL11

Crystal structure of SSL11 (PDB 2RDG) binding sialyl Lewis X (purple). The light blue coloured residues are involved in directly bonding to sLe\(^\alpha\) with the most important threonine and arginine residues shown. The red highlighted residues are identical and the yellow highlighted residues share similar properties, although not identical, as determined from an alignment of sequenced \textit{S. aureus} genomes. The asterisks provide orientation.

There are several clades of SSL11 reflecting the large variability in the amino acid sequence. This indicates there have been several points at which SSL11 has mutated. The SSL11 clades correlate with the CC families, showing that the conservation within these families and the evolution of SSL11 relates to that of the CC groups. The SSL11 proteins from non-human \textit{S. aureus}, which include: bovine (RF122, LGA251 and Newbould); murine (JSNZ) and ovine (ED133, SAO11) species, exhibited less homology with the human isolates. An exception is the avian SSL11 from isolate ED98 which has identity with the CC5 clade. The presence of SSL11 in non-primate species also suggests the importance of SSL11 to the bacterium.
Figure 3.2. Maximum Likelihood phylogenetic tree of SSL11

SSL11 proteins from sequenced \textit{S. aureus} isolates were obtained from the public NCBI database and aligned using Clustal Omega. The alignment was used to generate a Maximum likelihood phylogenetic tree using MEGA software. The SSL11 proteins are annotated by the strain they were determined from followed by the clonal cluster (CC) or sequence type (ST) if known. There is quite a lot of variability between the SSL11 alleles with multiple divergent branches. There is high homology between SSL11 from the same clonal complexes. Arrows indicate the alleles used in this study.
Chapter 3 – Analysing SSL11 interacting with myeloid cells

3.2.2. Purifying SSL11 alleles

SSL11 from *S. aureus* strains US6610, GL10, JSNZ and Newman were used for this project (see table 2.1 for strain details). These alleles are evolutionary diverse and found in different clades (see fig. 3.2). They therefore accommodate the variations in SSL11 observed between the *S. aureus* strains. An alignment of the alleles supports the observation that the β-grasp domain responsible for binding to sialyllactosamine is conserved when compared to the variability seen in the OB-fold (fig. 3.3).

As for SSL11US6610 and SSL11GL10, SSL11Newman, SSL11JSNZ and the SSL11US6610 R179A mutant were expressed as described previously (section 2.2.1) [117-119, 159]. In brief SSL11 was expressed as a fusion product (~45kDa) of SSL11 (~23kDa) and thioredoxin (~20kDa) containing six histidines. The tag allows the purification of the SSL11 from endogenous bacterial proteins by immobilised metal affinity chromatography (IMAC). The thioredoxin was removed by cleavage with reduced human rhinovirus 3C protease which cleaves at residues EVLFQ/GP [169]. The BamH1 site used in cloning is located downstream of the 3C protease cleavage site meaning all proteins expressed using the pET32a.3C plasmid contain a GSGP N-terminal overhang. SSL11US6610 wild-type, R179A mutant and JSNZ were directly

Newman STLEVRSQATQDLSEYYRNPFEEYTNQSKYEGKVTFTPNYQLIDVTLTGKEKQNFG-- 58
GL10 STLEVRSQATQDLKYKGGQSDLKNIGYEGKVTFTIHYPQISDVMGKEKEFNDD 60
US6610 STLEVRSQATQDLSEYYRNPFYDLRLNLSYREGNTVTVINHYQQTVDKLEKDKIDKID-- 59
JSNZ SRLAVTSKDTQELKYY5GTYFQNVSGYSENDELKNVFPGQLNVLGLSGLKDFKD-- 59

* * * *: **:*..**.   ::  * .** * ..:..    *   :.* *.:* .:

Newman EDISNVDFYVRENSDRSGNTASIGGITKTNQSNYIDKVNLITKNIDESVTSTSTSS 118
GL10 EEISSNIDFVVFYVVRENSRGITSNNGITYSNSQODINVKSVMQITKTSNNTSTTS 120
US6610 GNNENLDVFVYVREGSGQADNNISGGETNTRTQHIDTVQNVNVLVSKSQTGHTSTST 119
JSNZ GDYNGLDVFVYVREGSGQADNNISGGETKNKNTQFYVKNVEITPKFGHNTATQAE 119

*: ..*:****,.*... * **** :* *::*::*:* .* : :

Newman TYTINKEEISLKDPLKLHDKDLHNLKYTEPKDSKIRITMKGDFYFELNKKLQTH 178
GL10 YYYYKNSIELKDFKLKLDHKLHNLKYTEPKDSKIRITMKGDFYFELNKKLQTH 178
US6610 NYSIYKEEISLKDPLKLHDKDLHNLKYTEPKDSKIRITMKGDFYFELNKKLQTH 179
JSNZ TYSINKEEISLKDPLKLHDKDLHNLKYTEPKDSKIRITMKGDFYFELNKKLQTH 179

*: *::*:***********:::*:***************:::*::*:*:********

Newman MGDVIGDRNIEKVNL 195
GL10 MGDVIGDRNIEKVNL 197
US6610 MGDVIGDRNIEKVNL 196
JSNZ MGDVIGDRNIEKVNL 196

Figure 3.3. Alignment of SSL11 alleles used in this study

Alignment of SSL11 alleles from *S. aureus* strains Newman, GL10, US6610 and JSNZ. The residues involved in forming hydrogen bonds directly with sLe^x^ are highlighted in yellow and green and are highly conserved. Thr168 and Arg179 each are able to form 2 hydrogen bonds to the neuraminidase component of sialyllactosamine which when mutated completely lose binding capabilities.
Chapter 3 – Analysing SSL11 interacting with myeloid cells

passed over an anion exchange column to separate SSL11 and thioredoxin. Due to their pIs being similar to thioredoxin, SSL11\textsubscript{Newman} and SSL11\textsubscript{GL10} were first passed over IMAC (table 3.1). This step was required to remove as much thioredoxin as possible to decrease any contamination in subsequent purification steps. SSL11\textsubscript{Newman} and SSL11\textsubscript{GL10} were then subsequently passed over an anion exchange column. SSL11\textsubscript{US6610} and SSL11\textsubscript{JSNZ} have a similar pI to the buffer and were unable to bind the column, so were negatively selected from thioredoxin which binds the column (fig. 3.4). While not ideal, negative selection was preferred as it allowed complete separation of the proteins which was not efficient with positive selection at different pH values or when using cation exchange chromatography (data not shown). It also meant all SSL11 alleles were purified under the same conditions reducing variabilities during purification. SSL11\textsubscript{Newman} and SSL11\textsubscript{GL10} bind the column, and can be separated from thioredoxin by the percentage of salt required to elute the proteins as long as thioredoxin is not abundant (fig. 3.4). Typically SSL11 elutes with 300-500mM salt whereas thioredoxin is eluted at 600mM salt.

Table 3.1. Properties of the SSL11 alleles

<table>
<thead>
<tr>
<th>Protein</th>
<th>Theoretical pI</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSL11\textsubscript{US6610} WT</td>
<td>7.85</td>
<td>22.65</td>
</tr>
<tr>
<td>SSL11\textsubscript{US6610} R179A</td>
<td>7.01</td>
<td>22.57</td>
</tr>
<tr>
<td>SSL11\textsubscript{Newman}</td>
<td>5.98</td>
<td>22.30</td>
</tr>
<tr>
<td>SSL11\textsubscript{GL10}</td>
<td>5.50</td>
<td>22.54</td>
</tr>
<tr>
<td>SSL11\textsubscript{JSNZ}</td>
<td>7.79</td>
<td>22.20</td>
</tr>
</tbody>
</table>

The theoretical pI and size of SSL11 proteins as determined using the software ProtParam [170]. SSL11 alleles Newman and GL10 are more acidic than US6610 and JSNZ.
Chapter 3 – Analysing SSL11 interacting with myeloid cells

After the cleavage of the thioredoxin-SSL11 fusion protein with 3C protease the protein solution was dialysed to 20mM Tris.HCl pH 8.0. The thioredoxin was then separated and removed using anion exchange chromatography. A) Sensogram of SSL11US6610. SSL11 is found in the flowthrough (FT) whereas thioredoxin (thx) is eluted from the column with salt. SSL11JSNZ produces the same sensogram; B) SSL11GL10 has most of its thioredoxin removed by immobilised metal chromatography and exhibits one major peak when SSL11 is eluted. C) SSL11Newman also has most of its thioredoxin removed by IMAC but exhibits two major peaks. Subsequently it has been determined that peak 1 is a monomer of SSL11 while peak 2 is the dimer (see below). D) Silver stained non-reducing SDS-PAGE gel of fractions: i) SSL11US6610 FT; ii) SSL11Newman peak 1; iii) SSL11Newman peak 2 early fraction; iv) SSL11Newman peak 2 end fraction; v) SSL11GL10 peak; vi) SSL11JSNZ FT.
3.2.3. SSL11 dimer isolation

SSL11\textsubscript{Newman} contained two different forms as seen by the two peaks on the anion exchange column sensogram that required higher concentrations of salt for elution. This is most likely to be the dimer form as observed in the crystal structure of SSL11 [118]. The dimer is unlikely to alter the pI but would have an overall larger charge as there are two molecules of SSL11 and thus would require a higher salt concentration to elute. No observed dimer band was seen in a non-boiled, non-reducing SDS-PAGE gel (fig. 3.4d). However this does not exclude the presence of a dimer which may be transient. To confirm the existence of a dimer, recombinant SSL11 was passed over a Sephadex 75 10/300 GL size exclusion column which allows the separation of 3,000-600,000 globular units. The monomer, of ~23kDa was resolved from the dimer (~46kDa) for both SSL11\textsubscript{US6610} and SSL11\textsubscript{Newman}, but not SSL11\textsubscript{GL10} or SSL11\textsubscript{JSNZ} (fig. 3.5). The dimer eluted after 10mL of buffer was passed over the column while the monomer was detectable after 12mL. The dimer was approximately 2-fold less concentrated when compared to the monomer. As for the anion exchange column, the dimer had no observable band on a SDS-PAGE gel (only had the monomer band). When recombinant SSL11 is produced it exists solely as a monomer (data not shown). Within a week of purification, the dimer was detectable and could be separated from the monomer. This shows that SSL11 is able to dimerise spontaneously in solution without any external forces altering its structural conformation. Therefore, the more concentrated the SSL11 solution, the larger the proportion of the SSL11 dimer forms. The lack of dimer for SSL11\textsubscript{GL10} and SSL11\textsubscript{JSNZ} is likely to reflect a weaker affinity binding between the β7 strands and would thus require greater concentrations to form the dimer detectable by these chromatographic methods.
Size exclusion was used to separate the monomeric (M) and dimer (D) forms of SSL11 alleles: A) Newman; B) US6610; C) GL10 and D) JSNZ.
3.2.4. Biasensor analysis of SSL11 binding sialylated carbohydrates

Sialyl Lewis X (sLe\textsuperscript{x}) is a derivative of sialyllactosamine (sLacNAc) with an additional fucose moiety attached to the N-acetylglucosamine. SSL11 only forms one hydrogen bond to the fucose residue through the glutamic acid residue at position 170 [118]. This is in contrast to SSL4 which forms four hydrogen bonds to fucose [119]. For this reason, surface plasmon resonance was used to investigate the differences between SSL11 binding either BSA-sLe\textsuperscript{x} or BSA-sLacNAc. Both carbohydrates were bound at 100-200 response units (RU) to a CM5 chip using carbodiimide chemistry to ensure identical orientation. SSL11 as the analyte was passed over the carbohydrate surfaces at concentrations ranging from 0-30\,\mu\text{M} for 300 sec and the binding was measured using the Biacore T200. To guarantee that SSL11 existed in a single form, it was passed over the size exclusion column directly before analysing it in the Biacore. The separated monomer and dimer were passed over the chip. The dissociation constant (K\textsubscript{D}) was calculated using the Biacore T200 evaluation software by fitting the binding at equilibrium into a single-binding site model using the equation: 

\[
\frac{R_{eq}}{B_{max}} = \frac{[SSL11]}{K_D + [SSL11]} \]

where \(R_{eq}\) is the response at equilibrium, \(B_{max}\) is the maximal bound analyte at saturation, [SSL11] is the concentration of SSL11 and \(\frac{R_{eq}}{B_{max}}\) indicates the fraction of immobilised ligand that is bound.
Chapter 3 – Analysing SSL11 interacting with myeloid cells

3.2.4.1. SSL11 alleles binding sLe\(^x\)

SSL11 alleles from *S. aureus* strains US6610, GL10, JSNZ and Newman along with the mutant SSL11\(_{US6610}\) R179A were passed over a chip with immobilised sLe\(^x\) and their binding affinities were determined. SSL11\(_{US6610}\) R179A, even up to 8\(\mu\)M, was unable to bind the immobilised sLe\(^x\) as it loses its major hydrogen bonds to Sia (fig. 3.6). The dissociation constant for monomeric SSL11\(_{US6610}\) is 2.32 \(\pm\) 0.20\(\mu\)M compared to SSL11\(_{JSNZ}\) which had a \(K_D\) of 1.05 \(\pm\) 0.16\(\mu\)M; SSL11\(_{GL10}\) which had a \(K_D\) of 7.19 \(\pm\) 1.15\(\mu\)M and SSL11\(_{Newman}\) which had a \(K_D\) of 12.52 \(\pm\) 1.53\(\mu\)M (fig. 3.7a-d).

![Figure 3.6. Biacore analysis of SSL11\(_{US6610}\) R179A exhibiting no binding to sLe\(^x\)](image)

A concentration series ranging from 0-8\(\mu\)M of SSL11\(_{US6610}\) R179A was passed over immobilised BSA-sLe\(^x\) and the binding measured by surface plasmon resononance. A sensogram depicting binding responses (A) is fitted to a steady-state affinity binding model (B). SSL11\(_{US6610}\) R179A is unable to bind sLe\(^x\). Graphs representative of four experiments.
Figure 3.7. Biacore analysis of SSL11 alleles binding BSA-sLe\(^x\)

Alleles of SSL11 were passed over BSA-sLe\(^x\) for 300 sec to reach equilibrium and the responses recorded using surface plasmon resonance on the Biacore T200. Sensograms of binding responses against the concentration and steady-state affinity binding models of SSL11 A) US6610 (0-8µM); B) Newman (0-30µM); C) JSNZ (0-8µM) and D) GL10 (0-8µM). Sensograms representative of duplicate experiments.
3.2.4.2. SSL11\textsubscript{US6610} monomer compared to dimer binding sLe\textsuperscript{x}

The SSL11\textsubscript{US6610} monomer was separated from the dimer using size exclusion to produce two individual populations. These were then passed over a BSA-sLe\textsuperscript{x} surface using a doubling concentration series from 0.125 to 8\(\mu\)M. There is almost a 3 fold increase in affinity between the dimer and monomer where the \(K_D\) of the dimer is 0.86 ± 0.02\(\mu\)M compared to the monomer which has a \(K_D\) of 2.32 ± 0.20\(\mu\)M (fig. 3.8a-b). The dissociation curves between the different populations were also significantly different where the dimer did not dissociate from sLe\textsuperscript{x} and return to baseline as seen with the monomer (fig. 3.8c-d). SSL11\textsubscript{US6610} had to be made fresh to achieve pure monomer as within a week of production, the monomer would dimerise on the chip surface giving an intermediate \(K_D\) and the characteristic poor dissociation (data not shown).

![Figure 3.8](image)

**Figure 3.8.** SSL11\textsubscript{US6610} dimer demonstrates prolonged association with sLe\textsuperscript{x} when compared to the monomer

SSL11\textsubscript{US6610} size exclusion separated dimer (A,C) and monomer (B,D) were passed over BSA-sLe\textsuperscript{x} at equal concentrations (0-8\(\mu\)M) and the binding was measured. The calculated \(K_D\) for the dimer (A) and the monomer (B). The sensograms of the dimer (C) and monomer (D) are shown. The different dissociation curves are highlighted by the boxes. Sensograms representative of triplicate experiments
3.2.4.3. Biacore analysis of SSL11\textsubscript{US6610} binding sialylactosamine

SSL11\textsubscript{US6610} was passed over sLacNAc to examine if the fucose moiety contributed to the binding of SSL11 to sLe\textsuperscript{x}. BSA-sLacNAc was immobilised to a CM5 chip and 0-8\textmu M SSL11\textsubscript{US6610} was injected and allowed to adhere to the carbohydrate for 300 sec. Both the dimer and monomer of SSL11\textsubscript{US6610} were examined. The affinity of the dimer for sLacNAc was comparable to that of sLe\textsuperscript{x} with a K\textsubscript{D} of 1.27 ± 0.62\textmu M (fig. 3.9a). The affinity of SSL11 monomer for sLacNAc was 2.43 ± 0.18\textmu M which is the same as for sLe\textsuperscript{x} (fig. 3.9b). The dimer again had the different dissociation curve compared to the monomer as it did not return to baseline.

SSL11 dimer (A) and monomer (B) were passed over BSA-sLacNAc and the binding affinities were determined. The dissociation constants are comparable with the constants obtained when SSL11 is passed over sLe\textsuperscript{x}. The dimer once again did not dissociate entirely from the bound sLacNAc. Sensograms representative of triplicate experiments.
The dissociation constants for all samples are summarised in table 3.2. Attempts were made to calculate the dissociation constant of SSL11 binding sLe\(^x\) based on kinetic association (\(k_a\)) and dissociation rates (\(k_d\)). This was difficult for the monomer however as there was a large amount of variability, probably owing to the formation of the dimer on the chip surface (data not shown). This is evident by looking at the dissociation curves whereby dimer formation is more frequent at higher concentrations (fig. 3.8d). The dimer had similar concentration dependent variations, but was not as affected as the monomer. The calculated \(K_D\) for the SSL11 dimer binding sLe\(^x\) using kinetic analysis was 26.80nM ± 9.97nM. This was much greater than the calculated affinity (0.86 ± 0.02µM) and may reflect the altered dissociation curve for the dimer which remains attached for much longer.

Table 3.2. Summary of calculated steady state affinity SSL11 dissociation constants

<table>
<thead>
<tr>
<th>SSL11</th>
<th>(K_D) for sLe(^x) (µM)(^#)</th>
<th>(K_D) for sLacNAc (µM)(^#)</th>
</tr>
</thead>
<tbody>
<tr>
<td>US6610 monomer</td>
<td>2.32 ± 0.20</td>
<td>2.43 ± 0.18</td>
</tr>
<tr>
<td>US6610 dimer</td>
<td>0.86 ± 0.02</td>
<td>1.27 ± 0.64</td>
</tr>
<tr>
<td>JSNZ</td>
<td>1.05 ± 0.16</td>
<td>n.d.(^+)</td>
</tr>
<tr>
<td>GL10</td>
<td>7.19 ± 1.15</td>
<td>n.d.(^+)</td>
</tr>
<tr>
<td>Newman</td>
<td>12.52 ± 1.53</td>
<td>n.d.(^+)</td>
</tr>
</tbody>
</table>

\(^#\) data ± standard error; \(^+\) not determined; n=2 for binding sLe\(^x\); n=3 for binding sLacNAc
3.2.5. Flow cytometric analysis of SSL11 binding neutrophils

3.2.5.1. SSL11 competition for neutrophil receptors

As there was no observable difference between SSL11\textsubscript{US6610} binding to sLe\textsuperscript{x} and sLacNAc, it is conceivable that SSL11 could bind any sialylated ligand containing the core \(\alpha\text{2,3}\) sialylated galactose (Neu5Ac\(\alpha\text{2-3}\)Gal\(\beta\text{1-4}\)GlcNAc). A competition assay between labelled and unlabelled SSL11 was used to examine if SSL11 targets a single receptor or multiple sialylated receptors \[118\]. If SSL11 can bind any trisaccharide sialylactosamine, then once a specific receptor is saturated by SSL11, it could bind another sialylated receptor and so no competition would be observed. If this hypothesis was invalid, competition for the specific target receptor would be examined. Purified neutrophils were incubated with various concentrations of unlabelled SSL11\textsubscript{US6610}. Excess SSL11 was removed and the cells were labelled with 10nM fluorescein-SSL11\textsubscript{US6610} before being quantified by flow cytometry. As SSL11 is internalised into neutrophils, the experiment was repeated at 4°C to minimise any influence this may have on competition \[118\]. The gating strategy involved gating on the typical granulocyte population (fig. 3.10a). Of this granulocyte population, cell doublets were excluded by gating on linear cells in the forward scatter-area vs forward scatter-height dot plots. Typically, doublets weren’t seen in this population that would perturb results as SSL11-induced aggregates were larger and so had greater forward and side scatter and would be excluded from the initial granulocyte gate. The unlabelled SSL11 did not compete for receptors with the fluorescein-labelled SSL11 as the fluorescein (detected as FITC on the cytometer) signal peaks did not decrease despite a 50 fold excess of unlabelled SSL11 (fig. 3.10b-c). Higher concentrations could not be used as neutrophil aggregation would occur (discussed in 3.2.6). This confirmed the hypothesis that SSL11 can bind any core trisaccharide sialyllactosamine. This was true regardless of temperature, supporting the hypothesis as internalisation of SSL11 did not interfere with further SSL11 binding as other receptors are available.
Figure 3.10. No competition between fluorescein labelled - SSL11_{US6610} and unlabelled SSL11_{US6610} is observed showing SSL11 is able to bind multiple sialylated receptors

An equal amount or 50 fold excess of SSL11_{US6610} was incubated with granulocytes followed by treatment with 10nM SSL11_{US6610} labelled with fluorescein. Granulocytes were gated on using the strategy shown in (A) and measured using flow cytometry. Samples were prepared at either 37°C (B) or 4°C (C). Graphs representative of experiments done in triplicate.
Chapter 3 – Analysing SSL11 interacting with myeloid cells

3.2.5.2. Titration of SSL11 binding neutrophils

If SSL11 was able to bind a host of receptors on neutrophils, it also would not saturate until vast quantities are added. It was therefore expected that the amount of bound SSL11 would increase with higher concentrations of protein. This was examined by adding increasing amounts of SSL11\textsubscript{US6610} labelled with fluorescein to neutrophils. The samples were washed and examined by flow cytometry. Figure 3.11 demonstrates that SSL11 had increased binding to neutrophils with increasing concentrations and that saturation was not achieved. This lack of saturation was also observed with SSL4 which does not aggregate neutrophils [171]. 500nM was the maximal concentration that could be used as beyond this concentration SSL11 aggregates the neutrophils into dense clusters that block the flow through the cytometer. It is expected that the concentration would keep increasing until all available trisaccharide sialyllactosamine expressing receptors are saturated.

![Figure 3.11](image)

**Figure 3.11. Titration of fluorescein labelled SSL11\textsubscript{US6610} binding neutrophils exhibited no saturation**

Isolated neutrophils were incubated with increasing concentrations of fluorescein labelled SSL11\textsubscript{US6610} for 30 min at RT before being washed and analysed on the LSR II flow cytometer. Histogram representative of experiments done in duplicate.
3.2.6. SSL11 induced aggregation of neutrophils

The most notable feature of SSL11_{US6610} is that when added to neutrophils, the SSL11 caused the neutrophils to aggregate into dense clumps that are visible to the naked eye. It was hypothesised that this is a consequence of the SSL11 dimer crosslinking the cells together. This would be achieved by the excess SSL11 on the cell surface that has not internalised. To examine this, neutrophils treated with varying concentrations of the SSL11 alleles were compared by light microscopy.

3.2.6.1. Induction of neutrophil aggregation by different SSL11 alleles

Not all alleles dimerised readily in solution. Thus a titration of SSL11 concentrations was used to examine if aggregation would occur. Typically 1µM of SSL11_{US6610} induces aggregation. SSL11_{Newman}, which forms a dimer in solution, aggregated neutrophils at 1µM like SSL11_{US6610} (fig. 3.12). SSL11_{JSNZ} did not aggregate at 1µM, but did at concentrations greater than 6µM (fig. 3.12). SSL11_{GL10} did not cause aggregation at the concentrations tested (fig. 3.12). This supports the hypothesis that aggregation is caused by the SSL11 dimer. The SSL11_{US6610} R179A did not cause aggregation up to 10µM, showing that binding to cells and aggregation is carbohydrate dependent. CHIPS, which shares homology in the β-grasp domain structure and is able to bind neutrophils, also did not cause aggregation showing this is an effect caused by SSL11 (fig. 3.12).
Figure 3.12. Aggregation of neutrophils with SSL11 alleles

Neutrophils were incubated with SSL11 alleles or CHIPS and the resulting aggregation observed. All images are taken at the same magnification and represent the cell population from at least two individual experiments.
3.2.6.2. Comparison of neutrophil aggregation by monomer and dimer SSL11

SSL11_{Newman} was used to directly investigate what differences the SSL11 monomer and dimer have on aggregation. SSL11_{Newman} produces larger yields of both the monomer and dimer which can be separated when running over an anion exchange column. The monomer or dimer was then used to examine aggregation. As expected the dimer caused neutrophil aggregation and only required 1\mu M (fig. 3.13). The monomer however did not cause aggregation at 1\mu M, but did when the concentration was increased to 4\mu M and 8\mu M (fig. 3.13).

Figure 3.13. Neutrophil aggregation caused by SSL11 dimer

SSL11_{Newman} was passed over an anion exchange column and the monomer and dimer fractions collected. The SSL11 dimer was able to aggregate the neutrophils at the typical 1\mu M concentration. The monomer was titrated to examine induction of aggregation. Images represent triplicate experiments.
3.2.6.3. Aggregation of mononuclear cells

If the SSL11 dimer is responsible for aggregation, there should be no discrimination against which cells it can aggregate (as long as they are in suspension and can be bound by SSL11). For this reason human peripheral blood mononuclear cells and the THP-1 monocyte cell line were used to investigate aggregation caused by SSL11. SSL11\textsubscript{US6610} aggregated a select population of mononuclear cells (fig. 3.14). These are likely to be monocytes as SSL11 does not bind to B lymphocytes and only binds weakly to T lymphocytes [118]. This is supported by the observation that SSL11\textsubscript{US6610} is able to aggregate THP-1 monocytes (fig. 3.14). Again, SSL11\textsubscript{US6610} R179A did not cause aggregation as it is unable to bind the cell carbohydrates.

![Figure 3.14. SSL11 aggregation of mononuclear cells and THP-1 monocytes](image)

Isolated mononuclear cells or THP-1 monocytes were incubated with 1µM SSL11\textsubscript{US6610} or the R179A mutant for 45 min at RT. All images are taken at the same magnification and represent the cell population from at least two individual experiments.
3.2.7. SSL11 interaction with monocytes and macrophages

SSL11 has been shown to be capable of binding monocytes [118]. However binding to macrophages has not been investigated. Similarly internalisation into this cell lineage has not been examined.

3.2.7.1. SSL11 internalisation into monocytes

Mononuclear cells were isolated from healthy volunteers, stained with 0.25µM SSL11 US6610 labelled with fluorescein and imaged using confocal microscopy. However this method is not ideal as the monocytes are typically contaminated with lymphocytes. The THP-1 monocytic cell line was also used to represent a pure monocyte population. SSL11 bound and was internalised into peripheral blood monocytes (fig. 3.15a). The SSL11 localised into dense structures which matched with the observed localisation in neutrophils [118]. Surprisingly, despite strong binding, SSL11 was only weakly internalised into THP-1 monocytes with the majority remaining on the cell surface (fig. 3.15b) indicating that the leukemic THP-1 monocyte is missing a pre-requisite receptor or process required for SSL11 internalisation.

Figure 3.15. Live cell imaging of SSL11 internalisation into primary monocytes but not THP-1 cells

SSL11 US6610 labelled with fluorescein (green) was added to primary human monocytes (A) or THP-1 cells (B) at 0.25µM. Images are representative of the cell population from at least two individual experiments.
3.2.7.2. SSL11 internalisation into macrophages

Macrophages are tissue resident cells and exhibit properties dependent on their location. It is therefore difficult to get a representative population. To overcome this, a generic approach was used to generate macrophages with the most essential characteristics as determined by Daigneault et al [161]. The macrophages were then labelled with 0.25µM SSL11US6610-fluorescein and viewed using confocal microscopy. As for the monocytes, SSL11 was rapidly internalised into primary macrophages (fig. 3.16a) but not macrophages generated from THP-1 cells (fig. 3.16b). This again shows that a receptor or process is missing from THP1 cells that grant entry of SSL11 into the cells. The internalised SSL11 in primary macrophages is more fibrous and dispersed than seen in the monocytes and neutrophils. SSL11 also intensely stained the membrane protrusions.

![Figure 3.16: Live cell imaging of SSL11 internalisation into primary macrophages but not THP-1 derived macrophages](image)

SSL11US6610 labelled with fluorescein (green) was added to primary macrophages (A) or THP-1 derived macrophages (B) at 0.25µM. Images are representative of the cell population from at least two individual experiments.
Chapter 3 – Analysing SSL11 interacting with myeloid cells

A 3D reconstruction of the internalised SSL11 in macrophages was made using the Olympus Fluoview software. It demonstrates that SSL11 is traversing the entire cell in one large contiguous structure (fig. 3.17 and video 1 on attached CD). There are also major points on the cell surface of SSL11 staining where entry into the cells is achieved.

Figure 3.17. 3D reconstruction of SSL11 internalised into primary macrophages

A 3D construction was made using the z-series images of fluorescein-labelled SSL11US6610 (green) internalised into primary macrophages. See video 1 on attached CD for the rotating image. Arrow indicates concentrated surface staining at the top of the cell.

3.2.8. Biotin labelling of SSL11 bound cellular components

The Sulfo-SBED biotin transfer reagent is a chemical that contains four key constituents (fig. 3.18a). It contains an NHS ester group which reacts with amines allowing the attachment of the reagent to SSL11. A biotin is attached to a photoreactive aryl azide group, which upon UV photolysis, forms a nitrene that will react with any nearby nucleophile such as amines. This allows the covalent transfer of the biotin group to any protein that is proximally close to the sulfo-SBED labelled SSL11 which can then be detected and identified. Finally it contains a disulfide bond between the NHS ester group and aryl azide group allowing the reductive release of SSL11. This chemical is therefore ideal for examining protein to protein interactions and so was used to examine what SSL11 is binding.
3.2.8.1. Detecting biotin after incubation of Sulfo-SBED conjugated SSL11 with cells

When added to neutrophils, Sulfo-SBED conjugated SSL11\textsubscript{US6610} exhibits two bands at approximately 50kDa (fig. 3.18b arrow). Neither Sulfo-SBED conjugated SSL10 (provided by Ms W. Hou, fig. 3.18b lane 2) nor Sulfo-SBED conjugated recombinant CHIPS (fig. 3.18b lanes 3 and 4) produced a similar sized band showing it is SSL11 specific. The band was concentration dependent and increased with the amount of SSL11 added. The same ~50kDa bands were present when Sulfo-SBED conjugated SSL11\textsubscript{US6610} was added to primary macrophages (fig. 3.19a) and THP-1 monocytes (fig. 3.19b). Sulfo-SBED conjugated to the carbohydrate binding mutant SSL11\textsubscript{US6610 R179A} had a significantly diminished ~50kDa band as would be expected without any binding to the cells (fig. 3.19).

![Figure 3.18. Sulfo-SBED biotin tagging of SSL11 binding to neutrophils](image)

A) The Sulfo-SBED reagent with highlighted moieties. The NHS ester reacts with primary amines and so allows the conjugation of the reagent to SSL11. B) Western blot of neutrophils tagged with biotin following exposure to protein conjugated to Sulfo-SBED and UV illumination. The biotin was detected with streptavidin HRP. The lanes are 1) 0μM SSL; 2) 2μM SSL10; 3) 2μM CHIPS; 4) 4μM CHIPS; 5) 0.5μM SSL11; 6) 1μM SSL11 and 7) 2μM SSL11. Bands at approximately 50kDa (arrow) are specific to SSL11 (25kDa band). Gel representative of triplicate experiments.
Chapter 3 – Analysing SSL11 interacting with myeloid cells

Figure 3.19. Western blot of SSL11 biotin tagging of macrophages and THP-1 cells

A) PBMC-derived macrophages were incubated with Sulfo-SBED conjugated SSL11 and the transfer of biotin tags examined by Western probed with streptavidin HRP. Lanes are 1) 0µM SSL11; 2) 0.5µM SSL11; 3) 1µM SSL11; 4) 2µM SSL11 and 5) 2µM SSL11 R179A. B) THP-1 monocytes were treated as for the macrophages and the lanes include 1) 0µM SSL11; 2) 2µM SSL10; 3) 2µM SSL11 and 4) 2µM SSL11 R179A. Gel representative of triplicate experiments.

Based on the size, the most likely candidates for the ~50kDa band is the SSL11 dimer or the cytoskeletal monomers actin, vimentin or tubulin. Attempts were made to purify the biotin tagged proteins with streptavidin conjugated to agarose but results were inconsistent in purity. There were no observable bands on a SDS-PAGE gel (data not shown) so the area between 45kDa and 50kDa was excised from a gel and sent to the Centre for Proteomics, University of Auckland for identification by mass spectrometry. The area contained SSL11, actin and vimentin, confirming one of the bands is the SSL11 dimer with high confidence (see appendix 7.3). SSL11 interacting with actin and vimentin are discussed later.
3.2.8.2. Detecting the dimer of Sulfo-SBED SSL11 treated cells.

To confirm the presence of the dimer at ~50kDa, the Sulfo-SBED SSL11US6610 linked primary macrophage samples were run on a SDS-PAGE gel, transferred to nitrocellulose and probed with mouse monoclonal anti –SSL11. There was a band detected at ~50kDa with the anti-SSL11 monoclonal antibody in the sample that received SSL11US6610 only (fig. 3.20) confirming the band is the SSL11 dimer. There was a band present in all samples which is non-specific antibody binding.

![Image of gel with bands at 25kDa and 50kDa](image)

**Figure 3.20. Detection of SSL11 dimer in macrophages linked with Sulfo-SBED conjugated SSL11US6610**

PBMC-derived macrophages were incubated with SSL11 conjugated to Sulfo-SBED as previously and illuminated with UV. The cells were lysed, run on a reducing SDS-PAGE gel and transferred to nitrocellulose. The membrane was probed with mouse anti-SSL11 and then goat anti-mouse HRP. The lanes include 1) 0µM SSL11; 2) 2µM SSL11 and 3) 2µM SSL11 R179A. An SSL11 dimer is detected in only the wild type SSL11 lane as highlighted by the arrow. Gel representative of duplicate experiments.
3.2.8.3. SSL11 alleles forming dimers as detected by biotin transfer

To examine if all SSL11 alleles form the dimer on cells, the alleles were labelled with Sulfo-SBED and processed as before. All alleles showed the ~50kDa band indicating they are all able to form the dimer on the cells (fig. 3.21). There is an allelic difference in the strength of the signal reflecting the affinity of SSL11 for sialyllactosamine. These dimers could also be detected in the SSL11-Sulfo SBED solution when treated with UV (data not shown). The weak dimer band for SSL11GL10 likely reflects its weaker affinity for sLeX and lack of observable dimer in solution. Whereas SSL11 JSNZ has the highest affinity and so would concentrate more, aiding the dimerization on the cell surface.

Figure 3.21. SSL11 alleles dimerising on the surface of macrophages

Western blot of biotin tagged proteins of PBMC-derived macrophages treated with 2µM of SSL11 alleles conjugated with Sulfo-SBED biotin transfer reagent. The lanes are 1) No SSL11; 2) SSL11Newman; 3) SSL11US6610; 4) SSL11GL10; 5) SSL11JSNZ and 6) SSL11US6610 R179A. The dimer at ~50kDa is shown by the arrow with all alleles except the R179A containing the band to some degree. Gel representative of duplicate experiments.
3.2.9. Pulldowns of cell lysates with the SSL11 alleles

As the alleles bind sLe$^x$ with different affinities, it is appropriate to ask if they also have different binding patterns on cells. To address this, pulldowns of cell lysates were performed as proteins bound by SSL11$_{US6610}$ and SSL11$_{GL10}$ have been previously identified [122, 159]. SSL11 has been shown to pulldown lactoferrin and integrin $\alpha$M (CD11b) from granulocyte lysates and GpIIb/IIIa (CD41/CD61) from mononuclear lysates in a carbohydrate dependent manner. As can be seen from the gels, there are no observable differences between the SSL11 alleles binding either granulocytes (fig. 3.22a) or mononuclear cellular proteins (fig. 3.22b).

![Figure 3.22](image)

**Figure 3.22. SSL11 allele pulldowns of granulocyte and mononuclear cell lysates.**

SSL11 alleles conjugated to sepharose were used to bind and isolate proteins from A) granulocyte and B) mononuclear cell lysates. The samples were run on a 12.5% (A) or 10% (B) SDS-PAGE reducing gel and stained with coomassie. Previously identified bands are 1) Lactoferrin; 2) CD11b/Integrin $\alpha$M; 3) CD61 and 4) CD41. Gels are representative of triplicate experiments.
3.3. Discussion

A comparison of the multiple SSL11 proteins found in genome sequences from *S. aureus* reveals that SSL11 is quite diverse with identity only being shared at the carbohydrate binding site and residues wrapping around the protein from this site. It therefore seems likely the carbohydrate binding site is most important for SSL11, with strong selective pressures for this region along with maintenance of the tertiary structure. With such sequence diversity, it is difficult to imagine that SSL11, like SSL7 and SSL10, has multiple binding sites [140, 142, 145]. Several point mutations of conserved residues have been made (that would not interfere with the carbohydrate binding site) with no observable differences detected reflecting this (data not shown). It is hypothesised that the conserved residues around the carbohydrate binding site, such as the loop between the β6 and 7 strands along with the starting N terminal residues, confers selectivity for a particular sialylated receptor on the surface. Conservation of SSL11 from non-primate isolates supports the notion that SSL11 is important to the bacterium, yet it remains to be determined if SSL11 is able to bind sialyllactosamine or immune cells from these organisms. Sialic acid is a modification present in all higher animals (and some bacteria) with the structure of the carbohydrate depending on the enzymes available to the organism [54, 172]. For example sLe^x^ is not present on molecules from mouse liver due to the absence of fucosyltransferase which is required for the addition of the fucose moiety to sialyllactosamine [173, 174]. However there has been detection of sLe^x^ on PSGL-1 from mouse neutrophils, although at a much lower concentration when compared to humans [59, 175]. It is therefore postulated that SSL11 would be able to bind correctly sialylated molecules from the various species.

SSL11 forms a dimer as demonstrated when separated by size exclusion as previously seen in the crystal structure [118]. The dimer formed spontaneously in solution without the requirement for external forces or prior changes in structural conformation. Allelic differences were observed whereby SSL11 from strains Newman and US6610 readily dimerised in solution whereas GL10 and JSNZ did not. This most likely reflects differences in the affinities of the SSL11 proteins for themselves. Concentrating GL10 and JSNZ would most likely force the dimer to be formed. From crystallographic analysis, it appears that the dimer is formed by hydrogen bonding between two β7 strands of SSL11. For SSL11US6610, this is between residues 112-118 [118]. It has not been determined if the carbohydrate binding mutant R179A can dimerise, although it is expected it would as this mutation should not interfere with the β7 strand. Hydrogen bonds are mainly formed between the core amine
and carboxyl atoms rather than the side chains, allowing for diversity in these amino acids [118]. It is likely that the structure and length are most important as SSL11 is flat at the dimer interface. Variations to this may contribute to the affinity differences as seen in these SSL11 alleles.

It is hypothesised that dimerization is important for entry of SSL11 into cells. Many immune receptors get concentrated in lipid rafts on the membrane surface to aid in their function by allowing proximity to other molecules required for their activity [40, 176]. Because many glycosylated receptors are known to be sialylated, it has been estimated that concentrations of sialic acids can exceed 100mM equivalents due to being condensed into a small area [58, 177]. SSL11 would then be able to dimerise at these concentrations which would then bring two receptors into close proximity. The aggregated receptors would then be internalised as receptor aggregation is a mechanism used to cause signal transduction as it brings together domains required for these processes. For example, the Fc receptors are internalised following cross-linking of receptors by antibody:antigen complexes [178, 179]. Cross-linking of the integrin Mac-1, has been shown to induce its internalisation [180, 181]. SSL11 has been shown to be able to bind the CD11b domain of Mac-1 and so I hypothesise that Mac-1 grants SSL11 entry into the cell.

SSL11 alleles exhibit different affinities for sialyl Lewis X despite sharing the key residues required for hydrogen bonding to the carbohydrate. Newman was the weakest binder at 12.52 ±1.53µM which is tenfold weaker when compared to JSNZ with a dissociation constant of 1.05 ± 0.16µM. Although affinities all remain within the low µM range unlike the R179A mutant which has lost all ability to bind the carbohydrate. This is probably due to different conformations in stabilising the carbohydrate binding site. The SSL11US6610 dimer bound to sLeα with increased affinity. This increase however is not significant when considering there are now two SSL11 molecules. More importantly, the dimer did not dissociate from the carbohydrate as the monomer had done. This would suggest that the dimer may remain attached to the cell surface for a significant amount of time.

There was no observable difference in affinity for either the monomer or dimer of SSL11US6610 binding sLacNAc. This indicates the fucose is not critical in SSL11 binding. This lack of differing affinity is in contrast with SSL4 which has a greater affinity for both carbohydrates [119]. SSL4 also exhibits different affinities for sLeα (87.0 ± 4.5nM) and sLacNAc (471.7 ± 18.7nM), unlike SSL11. It has been demonstrated that this is because
SSL11 contains a histidine at position 178 whereas SSL4 has an asparagine at the equivalent position [119]. This histidine extends into the binding pocket disabling the closer association of the carbohydrate as seen in SSL4 [119]. SSL4 and SSL11 show no competition in binding granulocytes indicating they target different receptors [119].

Collectively these data shows that SSL11 does not discriminate between the carbohydrates, meaning SSL11 would bind anything with the core α2,3 sialylated galactose (Neu5Acα2-3Galβ1-4GlcNAc). It is believed that steric interference, other residues on the SSL11, or a combination would select for a specific receptor that can be internalised. Once this receptor is saturated by SSL11, then other correctly sialylated molecules could be bound. This is demonstrated by flow cytometry whereby SSL11 is unable to compete with itself and does not saturate the cells at the concentrations tested. These results are independent of SSL11 internalisation showing that the excess SSL11 is at the surface. This is reinforced by the ability of SSL4 to compete with itself as it displays differences in affinity [119]. There also appears to be more SSL11 bound in the competition experiment when the cells are pre-treated with higher concentrations of SSL11. This may be due to the remaining surface bound SSL11 being able to dimerise with the added labelled SSL11. Although it is difficult to say this conclusively since at the high concentrations, SSL11 aggregates the neutrophils resulting in variable population sizes which affects the flow cytometry results.

It was hypothesised that the observed neutrophil aggregation in the presence of SSL11US6610 was due to this promiscuous binding and crosslinking the cells by the dimer species. Such a hypothesis was supported by the finding that SSL11US6610 and SSL11Newman potently aggregated neutrophils whereas SSL11JSNZ (which showed the highest affinity for sLe\(^x\)) and SSL11GL10 required much higher concentrations to exhibit aggregation. This correlated with the finding that both SSL11US6610 and SSL11Newman readily formed dimers in solution whereas SSL11JSNZ and SSL11GL10 do not. It also demonstrates that aggregation is independent of glycan binding affinities. This was confirmed by the physical separation of stable dimer and monomer species with the former only showing aggregation at lower concentrations. It is believed that the monomer of SSL11Newman must first saturate the target receptor (and internalise) before crosslinking with other receptors on adjacent cells. Aggregation was not cell specific since monocytes (both PBMC and THP-1) were aggregated by SSL11US6610. Homotypic aggregation of neutrophils following chemotactic stimulation is an important aspect of neutrophil adhesion and capture by endothelial cells, allowing the neutrophils to slow down against the flow of blood [28, 182]. Aggregation is achieved mainly via L-selectin.
binding sialylated ligands on the adjacent neutrophil [28]. Mac-1 also plays a role in aggregation by binding ICAM-1 [28, 183]. It is hypothesised that SSL11 could be mimicking these receptors in cross-linking cells.

SSL11US6610 was shown to be internalised into primary monocytes and monocyte-derived macrophages isolated from blood. In contrast no internalisation was observed in unactivated THP-1 monocytes or macrophages derived from activated THP-1, indicating that some receptor or process is lacking or being interfered with in THP1 cells. The reason for this remains unknown. THP-1 cells express Fc receptors, TNF receptor, Mac-1, LFA-1, MHC receptors, CD14 and GM-CSF receptors, and so are not apparently missing any key immune receptors [184]. A 3D reconstruction of SSL11 internalising into macrophages shows SSL11 traversing the entire cells. Distinct regions on the membrane appear to be the site of SSL11 entry into the cell.

A biotin transfer tag was used in an attempt to identify the target receptor of SSL11. Two bands at approximately 50kDa were observed for SSL11 treated neutrophils, macrophages and THP1 cells. The 50kDa band was identified as the SSL11 dimer by mass spectrometry and an anti-SSL11 monoclonal antibody. This indicates that SSL11 forms dimers on the cell surface. Even SSL11JSNZ and SSL11GL10 alleles exhibited the 50kDa dimer indicating that this occurred independently of the ability of SSL11 to form dimers in solution. Moreover, the dimer was stable to boiling in 1% SDS under reducing conditions, suggesting that this dimer might be an important part of the mechanisms used by SSL11 to enter its target cell. No dimer band was observed for SSL11US6610 R179A since it could not bind the cells. However, like the other alleles, if the SSL11 R179A SBED-conjugated protein alone was exposed to UV, a dimer band could be seen (data not shown).

Pulldowns of PBMC and neutrophil lysates using the four alleles exhibited no observable differences. This shows that despite differences in affinity for the sialylated carbohydrate, there are no observable differences between the alleles binding cellular components.

SSL11 demonstrates complex interactions with glycosylated receptors on the cell surface. It is believed that SSL11 dimerisation clusters receptors in concentrated regions inducing its internalisation.
Chapter 4. Examining what effect SSL11 has on functional immune responses

4.1. Introduction

SSL11 has been shown to bind carbohydrates on FcαRI and PSGL-1 preventing their interactions with IgA and P-selectin respectively [118]. SSL5, the closest relative to SSL11, has also been shown to antagonise PSGL-1 binding to P-selectin [117, 153]. SSL5 also demonstrated the ability to activate platelets through binding to GpIIb/IIIa and GPVI, inhibit the activities of MMP9 and interfere with cells responding to chemokines [155-158]. SSL3, but not SSL11, has been shown to antagonise TLR-2, preventing its subsequent activities [150]. This chapter aims to investigate what effects SSL11 has on immune cell activities.

4.2. Results

4.2.1. Analysis of SSL11 and neutrophil chemotaxis

The conventional methods to examine the chemotaxis of neutrophils use a physical barrier such as agarose or a porous membrane which only allow the passage of actively migrating cells. However these methods are not suitable with SSL11 as it causes aggregation of the neutrophils which would block the spaces used by migrating neutrophils. For this reason, a chemotaxis assay was developed for the confocal microscope. Heat killed S. aureus were fixed to a glass confocal dish and opsonised with autologous serum to generate anaphylatoxins that would attract neutrophils that were treated or untreated with SSL11 US6610. In the absence of SSL11, the neutrophils migrated atop the surface phagocytosing any bacteria the cell encountered (fig. 4.1 and video 2). The direction of the neutrophils was dynamic, making the cell movement erratic. This is the major limitation of this assay as a defined chemotactic gradient cannot be achieved. However the neutrophils were clearly chemotactic and phagocytic only towards opsonised bacteria. When the neutrophils were pre-treated with SSL11 (for 10 min) the cells, while still able to phagocytose the bacteria, were no longer chemotactic and appeared tethered to the dish surface (fig. 4.1 and video 3). The neutrophils appeared to be tethered at the uropod. The membranes of the cells still exhibited the same erratic behaviour implying the cells were still able to sense the chemokine signals.
Figure 4.1. SSL11 treated neutrophils show diminished surface detachment when migrating to opsonised *S. aureus*

Neutrophils without (first 6 frames) or with (final 6 frames) 0.25µM SSL11L6610 migrating to serum-opsonised, heat-killed *S. aureus* containing GFP (green). The time in minutes are provided at the bottom right. The last frames for both samples (at 30 min) are a summary with the arrows indicating the migration path of the neutrophils. All samples were assessed at the same magnification. Images are representative of triplicate experiments.
Chapter 4 – Examining what effect SSL11 has on functional immune responses

4.2.1.1. SSL11 polarising to the neutrophil uropod during chemotaxis

To examine if SSL11\textsubscript{US6610} was localising to the uropod of a polarised neutrophil, the same experiment as in section 4.2.1 was performed with minor alterations. SSL11 labelled with Alexa 488 was used and \textit{S. aureus} did not contain the GFP vector. Figure 4.2a demonstrates how SSL11 staining was most intense at the uropod of an activated neutrophil (white arrows). SSL11 staining of the uropod is most likely due to the hypothesised unknown receptor polarising to this region as such polar staining was not observed in unactivated neutrophils (see ref [118] and fig. 5.1 for comparisons). There is evidence that SSL11 can associate with the surface of \textit{S. aureus} [185]. This was a concern for this experiment as SSL11 associated with the bacterium would polarise to the phagocytic membrane. To solve this possible problem, autologous serum was activated with heat-killed \textit{S. aureus} which was subsequently removed by centrifugation. The serum containing chemokines was then added to the neutrophils to observe where SSL11 localised to on the activated cell. SSL11 again exhibited greater staining at the uropod (fig. 4.2b). Under both conditions, SSL11 stained vesicles and structures could be seen originating from the uropod (fig. 4.2 red arrows, video 4).

![Figure 4.2](image.png)

**Figure 4.2. SSL11 concentrates to the uropod in activated neutrophils**

Polarised neutrophils were generated using activated serum \textbf{A}) containing or \textbf{B}) not containing heat killed \textit{S. aureus} and labelled with SSL11 conjugated to Alexa Fluor 488 (green). White arrows indicate the trailing uropod of the neutrophil. Red arrows highlight vesicles or structures originating from the uropod. Images are representative of the cell populations of triplicate experiments.
4.2.2. Neutrophil calcium signalling following activation

Altered signalling events following neutrophil activation may account for the observation that SSL11 halted migration of neutrophils. CHIPS, another *S. aureus* virulence factor that shares structural homology with the SSL β-grasp, inhibits signalling via the C5a receptor (CD88) and formyl peptide receptor (FPR) [63]. Mobilisation of calcium is a consequence of neutrophil activation with C5a and so could be used to assay if SSL11 interferes with signalling events [182, 186]. The fluorescent dye Fluo-3 is much more intense in the presence of calcium, so can be used to detect calcium fluctuations within the neutrophils (gated for granulocytes fig. 4.3a) when challenged with recombinant C5a (rC5a). Neutrophils were activated when challenged with rC5a, exhibiting almost a log fold increase in calcium (fig. 4.3b). If the neutrophils received SSL11US6610 there was a slight decrease in the amount of calcium mobilised, unlike CHIPS which completely inhibited calcium mobilisation (fig. 4.3b). The same was true when activated serum was added as a source of C5a (fig. 4.3c). CHIPS completely inhibited calcium mobilisation whereas SSL11 had no effect. SSL7 which blocks the release of C5a from C5 was shown to inhibit calcium mobilisation when added to the serum [139]. This indicates that SSL11 has no effect on neutrophil signalling events that result in a flux in calcium.
Figure 4.3. Calcium mobilisation following activation of neutrophils is inhibited by CHIPS but not SSL11

A) Gating on granulocytes. B) Neutrophils loaded with Fluo-3 were activated with 1nM recombinant C5a (rC5a) that was either untreated, or pre-treated with 0.5µM of either SSL11 US6610 or CHIPS. C) Similar experiment to (B) except activated serum was used as a source of C5a. The breaks in the plots indicate when the rC5a or activated serum was added. Dot plots are representative of three individual experiments.
4.2.3. Actin changes in activated neutrophils

Actin modifications were observed in 2D gels after neutrophils had been treated with SSL11 [122]. Changes in actin may relate to the inability of neutrophils to migrate in the presence of SSL11, since chemotaxis requires actin polymerisation and depolymerisation. However, no other evidence for the involvement of actin has been observed for SSL11 interacting with neutrophils. To examine if the activation state of the cell was important for SSL11 and actin, a Western blot was performed and probed with anti-β-actin. Neutrophils were either kept at rest in PBS or activated with fMLF using a concentration that had been shown to upregulate CD11b and shed CD62-L which are markers of activation (see appendix 7.3.6). The chemoattractant fMLF was used as it would not contaminate the sample with other serum proteins that would appear in the gel, unlike activated complement utilised previously. Lysate protein concentrations were determined using the Bradford assay so that equal amounts were loaded. This was confirmed by using an anti-vimentin antibody as a loading control. SSL11US6610 had no observable effect on actin under resting conditions (fig. 4.4 lanes 1 and 2). However when SSL11 was added to neutrophils before activation, there was a decrease in actin signal (fig. 4.4). This may reflect a loss in soluble globular-actin as it polymerises to filamentous actin. The SSL11 batch had been tested for LPS contamination which was below concentrations that would interfere with the experiment. The same results were obtained for neutrophils activated with the non-specific activator PMA (data not shown).

Figure 4.4. Activated neutrophils treated with SSL11 have altered actin when compared to non-treated cells

Lysates of resting (PBS) or activated (fMLF) neutrophils that were either untreated or pre-treated with SSL11US6610 were loaded at equal amounts and separated by SDS-PAGE. This was transferred to a membrane, probed with anti-β-actin and detected. The membrane was stripped and then probed with anti-vimentin. Images are representative of three individual experiments.
4.2.4. Analysis of SSL11 on the viability of neutrophils

4.2.4.1. Induction of cell death in the presence of SSL11

Bacterial toxins may intoxicate their host cells resulting in cell death. For example, the *Bordetella pertussis* adenylate cyclase toxin induces the generation of pathological quantities of cyclic AMP [187]. To examine if SSL11 causes cell death in neutrophils, it was incubated with cells for up to 2 hours. Propidium iodide was used as a marker for membrane integrity, a sign of cell death. Ethanol was used to disrupt the membrane as a positive control. Table 4.1 demonstrates that there was only a slight increase in cell death induced by SSL11, as seen by similar numbers of PI positive cells (1-2% without compared to 4-5% with SSL11) within the two hours. However this did not change over the course of the experiment and is believed to have arisen from the higher mechanical force required to resuspend SSL11 aggregated neutrophils. While the geometric mean was also slightly higher in SSL11 treated cells, this would relate to the higher number of PI positive cells. Neither population was comparable to the positive control which exhibited much greater PI staining. This does not exclude the possibility that death may be induced by SSL11 after longer times. However this would need to be examined using alternative non-adherent cells as neutrophils will start to become apoptotic after a few hours following the isolation from blood.

**Table 4.1 Neutrophil cell death in the absence of presence of SSL11**

Time course of 0.25μM SSL11 incubated with neutrophils. Cell death examined by proportions and intensity of cells stained with propidium iodide (PI). Data representative of duplicate experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage of total cells that are PI positive</th>
<th>Geometric mean of PI staining (mfi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils 0 min</td>
<td>1.28</td>
<td>88.8</td>
</tr>
<tr>
<td>Neutrophils with SSL11 0 min</td>
<td>4.41</td>
<td>225.0</td>
</tr>
<tr>
<td>Neutrophils 30 min</td>
<td>1.72</td>
<td>98.9</td>
</tr>
<tr>
<td>Neutrophils with SSL11 30 min</td>
<td>4.85</td>
<td>221.0</td>
</tr>
<tr>
<td>Neutrophils 60 min</td>
<td>1.66</td>
<td>95.5</td>
</tr>
<tr>
<td>Neutrophils with SSL11 60 min</td>
<td>4.77</td>
<td>224.0</td>
</tr>
<tr>
<td>Neutrophils 120 min</td>
<td>1.31</td>
<td>88.4</td>
</tr>
<tr>
<td>Neutrophils with SSL11 120 min</td>
<td>4.6</td>
<td>204.0</td>
</tr>
<tr>
<td>Ethanol treated neutrophils</td>
<td>61.9</td>
<td>3262.0</td>
</tr>
</tbody>
</table>
4.2.4.2. Analysis of neutrophil apoptosis in the presence of SSL11

*S. aureus* has been shown to modulate apoptosis of neutrophils [188, 189]. PVL has demonstrated the ability to target the mitochondria to induce NFκB signalling and apoptosis [190, 191]. Staphopain B, which cleaves CD11b, has been shown to also induce apoptosis [192]. The ability of SSL11 to induce apoptosis was investigated using flow cytometry. Propidium iodide (PI) was used to stain necrotic cells and fluorescein labelled annexinV, which binds phosphatidylserine, was used to examine apoptotic cells [193]. UV irradiation was used to generate apoptotic neutrophils as a positive control. This positive control had diminished cell numbers owing to the eventual destruction of the cells and so in most preparations had low numbers of necrotic cells. SSL11US6610 caused a slight increase in annexinV positive cells (from 0.384 to 4.28) but this population was never much greater than the resting cells (fig. 4.5). The same was true for the necrotic population which only slightly increased with SSL11 (0.015 to 4.770). However there was no apparent loss in cell numbers and this slight increase in necrotic cells is again believed to have arisen from the greater mechanical stress involved in resuspending SSL11 treated cells. A greater proportion of AnnexinV positive cells and some cell loss would be expected if apoptosis was being induced by SSL11.
Figure 4.5. SSL11 does not induce neutrophil apoptosis

Neutrophils were dual labelled with propidium iodide and fluorescein conjugated AnnexinV. Cells were treated with either PBS or 0.5µM SSL11 for 30 min. Some cells were irradiated with UV to induce apoptosis as a positive control. Results are representative of triplicate experiments.
Chapter 4 – Examining what effect SSL11 has on functional immune responses

4.2.5. SSL11 and complement

SSL7 has been shown to inhibit the activity of complement through the prevention of C5 cleavage [135, 137, 140]. Complement proteins are glycosylated with a wide variety of N-linked sugars that include sialylated termini [194]. SSL11 has been shown to bind C4, however it is unknown if it is able to perturb the complement responses [143]. To examine if SSL11 can inhibit complement, a variety of assays were performed.

4.2.5.1. Complement-mediated haemolysis of erythrocytes

A total complement assay (which has bias toward the classical pathway) that utilises lysis of red blood cells as a marker of complement activity, was performed with SSL11. Guinea pig serum provided the complement to the assay. Figure 4.6 demonstrated that no SSL11 allele had any effect on complement activity.

![Figure 4.6](image)

Figure 4.6. None of the SSL11 proteins have any effect on total complement activity

IgG opsonised sheep red blood cells were lysed by complement present in guinea pig serum allowing for an absorbance reading at 412nm for haemoglobin. Water lyses the cells through osmotic pressures providing a maximal reading for lysis. The buffer only controls for spontaneous lysis. Each condition was done in duplicate and repeated twice. Data points are plotted as mean ± standard deviation.
4.2.5.2. SSL11 in human complement ELISAs

The total complement assay uses guinea pig serum as a source of complement. SSL7 and SSL10 have exhibited species specificity towards C5, IgA and IgG [135, 142]. It is therefore plausible that SSL11 may not bind essential components in guinea pig serum and so no effect would be observed. Human complement ELISAs were performed that detect the formation of the MAC (C5b-9) as a marker of complement activation. Human serum was used as the source of complement in these ELISAs. Heat-aggregated IgG was used to mimic the antibody-antigen complex for the classical pathway, heat-killed S. aureus was used to activate the alternative pathway and mannan was used to activate the lectin pathway. For the alternative pathway ELISAs, EGTA was included to inhibit classical and lectin pathway activation. As the alternative pathway requires more serum, higher concentrations of SSL11 were used. Only a minor effect was seen with SSL11 in all three pathways when compared to SSL7 (fig. 4.7). Inhibition was greater in the classical and lectin pathways which likely reflects the ability of SSL11 to bind C4 which is not required in the alternative pathway. However, high concentrations of SSL11 were needed to have an effect, which is unlikely representative of physiological conditions. The carbohydrate binding mutant, R179A was included in the classical and lectin pathways as there was slight inhibition in these assays. The SSL11 R179A mutant exhibited similar levels of inhibition at the highest concentration demonstrating inhibition is independent of carbohydrate binding in these pathways.
Figure 4.7. SSL11 has no inhibitory activity in any of the human complement pathways

SSL11 in human serum was added to plates coated with antigen required to activate the classical, lectin and alternative complement pathways. Complement was detected using an anti-C5b-9 antibody as a marker of end stage complement activity. Background was measured from serum and buffer added to uncoated wells which was subtracted from all samples. Each condition was done in duplicate and repeated twice. Data points are plotted as mean ± standard deviation.
4.2.6. SSL11 and platelet coagulation

SSL10 has been shown to delay calcium-dependent clot formation [143]. SSL5 has demonstrated the ability to interfere with platelet coagulation due to binding of platelet glycoproteins GpIIb/IIIa and GPVI [155, 156]. SSL11 can also bind GpIIb/IIIa as observed in mononuclear cell pulldowns [159]. To examine if SSL11 can also interfere with the coagulation cascade, platelet rich (PRP) and platelet poor plasma (PPP) was used. Calcium-dependent coagulation was induced with calcium, causing fibrin clots and increased solution turbidity. Platelet activation was examined using ADP, which causes platelet aggregation and decreases solution turbidity [195]. Therefore both processes could be measured using optical density. SSL11 US6610 had no effect on the initiation, duration or intensity of calcium-induced clot formation in PPP or PRP (fig. 4.8a and b). Similarly, SSL11 US6610 exhibited no effect on platelet aggregation when activated with ADP (fig. 4.9c). SSL11 was titrated in all experiments but exhibited no differences to the concentrations shown in figure 4.8 (data not shown).
Figure 4.8 SSL11 does not alter coagulation of plasma

Calcium induction of the coagulation cascade was examined over time in either A) platelet-poor plasma (PPP) or B) platelet-rich plasma (PRP). C) Platelet activation and aggregation, in PRP, was induced by ADP and examined over time. SSL11 was added to the plasma, where necessary, prior to the addition of calcium or ADP. Each condition was done in duplicate and repeated twice. Data points are plotted as mean ± standard deviation.
4.2.7. Analysis of SSL11 cellular activity by mass spectrometry

To examine if there were global protein changes in neutrophils as a consequence of SSL11 activity, a pilot study using gel-free proteomics was used. This was performed using both resting and fMLF or PMA activated cells (section 2.2.7.3). Cell lysates were sent to Dr Frank Schmidt from the Applied Proteomics Group from the Ernst Moritz Arndt University in Germany for measurement and analysis. Samples were measured three times and data normalised using median experimental driven tendency normalisation as well as robust Z-score normalisation. Global comparisons of protein intensities from all samples are shown in figure 4.9. The most notable difference was that the protein cell cycle and apoptosis regulator (CCAR1) was increased in the neutrophils that had received SSL11, PMA and SSL11 and fMLF. CCAR1 is a transcriptional regulator that recruits important transcriptional mediators to the gene promoter [196]. Another noticeable difference was the absence of the Furry homolog-like protein (FRYL) and flap endonuclease 1 (Fen1) from SSL11 treated and fMLF activated neutrophils.

Figure 4.9. Global analysis of neutrophil protein amounts

Resting neutrophils (RN); PMA activated neutrophils (PN) or fMLF activated neutrophils (FN) were lysed and the protein levels determined by liquid chromatography mass spectrometry. Samples were measured three times. Spots represent individual proteins with the height of the stalk indicating abundance. Notable changes are highlighted in the boxes. Created by Dr. F. Schmidt.
Chapter 4 – Examining what effect SSL11 has on functional immune responses

Fen1 is responsible for removing 5’ overhanging nucleotides during replication and repair [197]. FRYL has been shown to perform as a transcriptional activator and is also proposed to regulate actin based on similarity [198]. However, changes in the less abundant proteins are more challenging to observe. A 3D principal component analysis (PCA) plot is used to capture dominant structures within the samples by accounting for as much variability as possible (fig. 4.10). Deviation from the centre of the plot indicates a greater difference of the protein in the various samples when considering all variables. Two notable proteins that played a role in the formation of the PCA plot were the lysosomal trafficking regulator (LYST) and protein scribble homolog. LYST has been shown to be involved in regulating maturation of the phagosome and autophagosome in trafficking to the lysosome [199, 200]. The protein scribble homolog has been shown to be an important regulator of cell polarisation and migration via PAK signalling [201-203]. The significance of these proteins and others, in the activity of SSL11, remains unknown and requires further examination.

Figure 4.10. Principle component analysis of SSL11 treated and untreated neutrophil protein amounts.

Three dimensional PCA plots showing dominant proteins identified from the various samples. The lysosomal trafficking regulator (LYST) and protein scribble homolog (Scrib) which contributed largely to the generation of the plot are shown. Created by Dr. F. Schmidt.
4.3. Discussion

With the full activity of SSL11 unknown, this chapter aimed to try to identify the functional effects SSL11 has on neutrophils. SSL11 had previously been shown to inhibit P-selectin mediated neutrophil rolling \textit{in vitro}, and so it was desirable to examine what effect SSL11 has on neutrophil chemotaxis [122]. SSL11 inhibited the migration of neutrophils to surrounding opsonised \textit{S. aureus}. Despite the neutrophils showing dynamic motion, it appeared they were tethered at the uropod as this pole could not be detached. It has yet to be determined if the reduced migration was a result of SSL11 altering cellular responses or if it was crosslinking some cellular structures. Halted migration was not caused by SSL11 antagonising chemotactic receptors, in a similar manner to CHIPS, as SSL11 did not inhibit rC5a or \textit{S. aureus} challenged serum from activating neutrophils as observed by calcium mobilisation. It is also unlikely that signalling events are affected as the neutrophils were still clearly responsive to adjacent opsonised bacteria despite being unable to migrate.

Fluorescently labelled SSL11 intensely stained the uropod of activated neutrophils. This may suggest that the halted migration was caused by the actions of SSL11 at the uropod. Vesicles and structures could be seen originating from the uropod, supporting the hypothesis that SSL11 dimers, concentrated at a defined surface area, are internalised by induction of endocytosis. Several receptors that polarise to the uropod have been characterised which are generally involved in adhesion [40]. Leukosialin (CD43) is found on granulocytes, monocytes and T cells and is heavily glycosylated with sialylated \textit{O}-linked carbohydrates [204, 205]. It has also been linked to aiding in anti-adhesive functions at the uropod as crosslinking CD43 receptors with antibodies induced neutrophil locomotion [206]. CD157, a GPI-linked glycoprotein, polarises to the uropod and is involved in actin remodelling and cell shape [207]. CD157 is glycosylated with multiple \textit{N}-linked sugars of unknown composition [208]. Mac-1 is rapidly polarised to the uropod [31]. L-selectin which is involved in neutrophil adhesion and aggregation and PSGL-1 both polarise to the uropod [25]. E-selectin binding sLe\(^\alpha\) on L-selectin and PSGL-1 drive their locomotion to the uropod along with strengthening the binding of Mac-1 to ICAM-1 [25]. These receptors are all contained in rafts or the immune synapse which, at the uropod, is defined by having the sialylated glycolipid GM1 [40, 43]. This would provide a heavily concentrated region of sialylated glycoproteins or glycolipids for SSL11 to bind and dimerise. It is possible to hypothesise how SSL11 binding these receptors could interfere with migration as they are all involved in regulating attachment and detachment. SSL11 has been shown to bind PSGL-1 which could be
mimicking E-selectin induced migration to the uropod [118]. SSL11 binds to Mac-1 and its polarisation to the uropod supports the hypothesis that Mac-1 may be involved in the internalisation of SSL11 [122, 159].

SSL11 had no observable effect on phagocytosis. As SSL11 is capable of binding Mac-1, phagocytosis would need to be explored further as Mac-1 provides a significant role in phagocytosis by associating with the FcγRIII and binding C3bi [20, 209].

SSL11, when added prior to activating cells, reduced the amount of soluble β-actin observed on a Western blot. It is yet to be determined if there is a reduction in available actin epitopes or if there is a modification to the actin that removes epitopes or makes the actin less soluble (for example polymerisation). Based on 2D-gels performed by Dr M. Chung, the modification of actin is possible [122]. The above mentioned receptors that are polarised to the uropod suggest it is possible that SSL11 binding and internalisation would act upon the actin regulators or actin itself. Several AB toxins alter actin responses by the addition of ADP-ribose to a protein involved in actin remodelling [210]. The C3 exotoxin from Clostridium botulinum and cytotoxic necrotizing factor 1 (CNF1) from E. coli both ADP-ribosylate Rho, a GTPase that is a master regulator of actin [211, 212]. Despite ADP-ribosylating the same molecule, the outcome is different [212]. SSL11 is unlikely to be an ADP-ribosyltransferase which could account for these actin changes (data not shown). While the active sites for ADP-ribosyltransferases are poorly defined, SSL11 does not appear to contain the nucleophilic arginine in proximity to a conserved glutamic acid [213, 214].

SSL11 did not intoxicate neutrophils to cause cell death within two hours. Analysis of SSL11 in a cell line, permissive of its internalisation, would prove useful in allowing for greater time periods to be examined. This would provide conclusive evidence to support this finding. There was a slight induction of neutrophil apoptosis. However this appears insignificant when compared to toxins such as anthrax lethal factor and B. pertussis adenylate cyclase or Staphopain B from S. aureus which cleaves CD11b and induces apoptosis in neutrophils [192, 215, 216].

SSL11 had no effect in the total complement assay. It had a minor effect on MAC formation in the human complement ELISA. The greatest inhibition of complement activation was seen in classical and lectin pathways. This is most likely due to the ability of SSL11 to bind C2 and C4 [143]. However this effect was several orders of magnitude less significant than the
Chapter 4 – Examining what effect SSL11 has on functional immune responses

effect of SSL7. Such high concentrations are not physiological and are unlikely to be encountered in vivo.

Unlike SSL5, SSL11 did not appear to have any effect on the calcium-dependent coagulation cascade or activation and aggregation of platelets. This may reflect differences between the two SSLs, especially when considering that SSL5 is more positively charged when compared to SSL11. SSL11 is able to bind GpIIb/IIIa, an integrin that recognises von Willebrand Factor (vWF), which is exposed when endothelial cells are damaged [29, 217]. Interactions between vWF and GpIIb/IIIa induce outside-in signalling which changes the conformation of GpIIb/IIIa allowing it to bind fibrinogen with high affinity [29, 217]. Platelets and fibrinogen form a clot to contain pathogens and open wounds. Platelets and endothelia express P-selectin, the ligand for PSGL-1, allowing neutrophils and platelets to interact and contain these cells to the damaged site [29, 217]. It is therefore plausible that SSL11 could aggregate neutrophils and platelets by crosslinking these glycosylated receptors.

A pilot proteomic study of the changes SSL11 induces in neutrophils revealed several regulators of transcription and endocytic trafficking. These may relate to the observed locations of SSL11 within the cell as discussed in the next chapter. Further investigation into trafficking events following SSL11 treatment would provide clearer details and associations with SSL11 activity. More targeted mass spectroscopy following subcellular fractionation would be suitable to examine these changes [218].

Unfortunately, the direct activities of SSL11 still remain unknown. However, SSL11 is able to inhibit neutrophil migration following polarisation to the uropod. There also appears to be activity in activated neutrophils not seen in resting neutrophils which would be more beneficial to the bacteria.
Chapter 5. Examining internalised SSL11 in myeloid cells

5.1. Introduction

SSL11 has been shown to internalise into neutrophils at 37°C but not 4°C [118]. One possible route of entry for SSL11 would be by directly translocating across the cell membrane. This is an unlikely route as the temperature dependence indicates an active internalisation requiring cellular machinery. However it cannot be excluded as the adenylate cyclase toxin from *B. pertussis* translocates directly across the membrane of erythrocytes, but requires a temperature-dependent post-translational modification [219]. The most plausible entry of SSL11 into cells would be via an endocytic mechanism which includes: phagocytosis; macropinocytosis; or receptor-mediated endocytosis (clathrin- or caveolin-dependent) [220].

While none of the SSL entry mechanisms have been investigated, some clues may be gleaned from a family of toxins known as the AB toxins [221]. This family includes many notable intracellular toxins including: anthrax; diphtheria; cholera; shiga; botulinum and tetanus which are able to enter their target cells via different mechanisms [221]. For example anthrax protective antigen binds the anthrax toxin receptor (ATR) and is internalised into endosomes using clathrin-dependent receptor-endocytosis [222-225]. In contrast cholera toxin binding GM1 is found in caveolae and so internalises via caveolin-dependent receptor endocytosis [226, 227]. Although there is evidence that clathrin also can be used for the internalisation of cholera toxin [228]. Similarly Shiga toxin which also binds a carbohydrate, Gb3, enters cells via clathrin- rather than caveolin- dependent endocytosis [229-231]. Ricin, the plant AB toxin which binds both glycoproteins and glycolipids containing terminal galactose, enters cells using a variety of endocytic mechanisms due to the large variation in receptors [231-233]. In this chapter, the internalisation of SSL11 was investigated to determine the route of entry and to where it is directed.
Chapter 5 – Examining internalised SSL11 in myeloid cells

5.2. Results

5.2.1. Internalised SSL11 in neutrophils

SSL11 from various *S. aureus* alleles exhibited different affinities towards sLe^x^ (see table 3.2). It was hypothesised that SSL11 dimerises on the cell surface in rafts containing concentrated sialylated glycoproteins. As they are concentrated on the cell surface, it was postulated that all alleles should internalise despite the ten-fold differences in affinity. Similarly the monomer fraction should be internalised as the monomer could dimerise on the cell surface.

5.2.1.1. SSL11 allele internalisation into myeloid cells

SSL11 alleles were labelled with Alexa Fluor 488 at the same time to accommodate for technical variations. The alleles have similar numbers of lysines with SSL11_{GL10} containing 22; SSL11_{JSNZ} having 20 and both SSL11_{Newman} and SSL11_{US6610} containing 19. They should therefore, be approximately equal in labelling intensity. However when calculating the degree of labelling, SSL11_{JSNZ} was under-labelled compared to the other alleles. SSL11_{JSNZ} had 0.33 moles of dye per mol of protein compared to 0.65 mol for SSL11_{Newman} and 0.9 mol for SSL11_{GL10} and SSL11_{US6610}. Therefore the amount of internalised SSL11 protein cannot be directly compared using fluorescent intensities. Despite the intensity difference, all alleles were observed internally in both neutrophils (fig. 5.1) and primary macrophages (fig. 5.2). SSL11_{US6610} R179A was unable to bind cells due to the loss in carbohydrate binding (fig. 5.1). In neutrophils, SSL11 was typically found in distinct patches (fig. 5.1). When aggregates were observed, there was intense staining between the cells supporting the finding that the SSL11 dimer crosslinks the cells (fig. 5.1 arrow). The SSL11_{US6610} and SSL11_{Newman} alleles, that dimerise readily in solution, have intense staining of the membrane when compared to SSL11_{JSNZ} and SSL11_{GL10}. This is unlikely to be an influence of the different degrees in labelling as SSL11_{GL10} and SSL11_{US6610} were labelled to a similar degree. In macrophages the staining is more dispersed and localises to a larger region close to the nucleus (fig. 5.2).
Figure 5.1. Internalisation of SSL11 into neutrophils

Internalisation of 0.25μM SSL11 alleles (green) into neutrophils was achieved by a 30 min incubation at RT. Arrow depicts a neutrophil aggregate. Scale bars are all 5μm with images representing approximately the centre of the cell based on a z-series. Images are representative of three repeat experiments.
Figure 5.2. Internalisation of SSL11 into primary macrophages

Internalisation of 0.25μM SSL11 alleles (green) into macrophages was achieved by a 30 min incubation at RT. Arrows demonstrate perinuclear SSL11 staining. Scale bars are all 10μm with images representing approximately the centre of the cell based on a z-series. Images represent duplicate experiments.
Chapter 5 – Examining internalised SSL11 in myeloid cells

5.2.1.2. SSL11 monomer internalisation into neutrophils

SSL11\textsubscript{US6610} was labelled with fluorescein before being passed over a size exclusion column to separate the monomer from the dimer. The free fluorescein did not interfere with the separation of the monomer as it elutes much later owing to its smaller size (fig. 5.3a). The monomer was then directly added to neutrophils at 0.25µM and used for imaging of its internalisation. The monomer is internalised into neutrophils into the typical punctate structures (fig. 5.3b). The signal is not as intense owing to the diminished photostability of fluorescein compared to Alexa Fluor 488 which was used to get enough labelled monomer.

**Figure 5.3. SSL11 monomer internalised into neutrophils**

A) Sensogram of SSL11\textsubscript{US6610} labelled with fluorescein being separated by size exclusion to isolate the monomer. Excess fluorescein eluted after the monomer; B) The SSL11\textsubscript{US6610} monomer (green) was then added to neutrophils at 0.25µM and imaged. The experiment was done in duplicate.
5.2.2. Modification of SSL11 within cells

It is possible that SSL11, once inside the neutrophil, could be modified by cellular components to exert its effect. A likely modification is cleavage of SSL11 by cellular proteases. A number of AB toxins require proteolytic digestion using host proteases to function [234]. For example anthrax protective antigen requires cleavage by furin at the cell surface to facilitate its oligomerisation [225]. To investigate this, biotinylated SSL11<sub>US6610</sub> was incubated with neutrophils and analysed by Western blot to observe if the SSL11 band decreased in size. There was no reduction in size of SSL11 indicating that it is unlikely to be cleaved within the cell (fig. 5.4). This method does not consider proteases from <i>S. aureus</i> or cleavage at the termini which would release small peptides. If evidence for any modifications are subsequently identified, these issues will need to be addressed.

![Western blot](image_url)

**Figure 5.4. Western blot demonstrates that SSL11 is not cleaved within neutrophils**

Biotin labelled SSL11 incubated within neutrophils for 45 min and detected by streptavidin conjugated to HRP. Image representative of triplicate assays.
5.2.3. Subcellular fractionation of granules from neutrophils treated with SSL11

Neutrophils are highly granular which allows them to respond immediately to any threats as these granules contain molecules required for their function [235]. There are four major classes of granules as defined by the abundance of certain proteins contained within the granules: azurophilic (contain myeloperoxidase); specific (abundant in lactoferrin); gelatinase (abundant in gelatinase) and secretory (abundant in albumin) [235]. SSL11 is able to co-purify with lactoferrin and SSL5 has been shown to inhibit the activity of gelatinase [122, 157]. For these reasons, the possibility that SSL11 could have an activity on these molecules was investigated using subcellular fractionation to determine if they are present at the same location within a cell.

Neutrophils were incubated with SSL11US6610 and then broken by nitrogen cavitation. The individual neutrophil granules were then separated into layers by high speed centrifugation using a Percoll gradient. Fractions of the layers were then collected from the base of the tube. SSL11US6610 conjugated to biotin was used so its location within individual fractions could be observed using streptavidin conjugated to HRP. SSL11 was present in all fractions starting from fraction 13 with the greatest amounts being present in the later fractions (fig. 5.5). Based on the publications this method was developed from, it is estimated that SSL11 is detectable with gelatinase and secretory granules. [163-165].
Chapter 5 – Examining internalised SSL11 in myeloid cells

Figure 5.5. Western analysis of neutrophil subcellular fractionation of neutrophils containing SSL11

Western blot of granule fractions from the subcellular fractionation of neutrophils by centrifugation through a discontinuous sucrose gradient, probed with streptavidin to detect SSL11 conjugated to biotin. Fraction numbers are shown for each well with decreasing density from left to right (1-29). Blots are representative of two individual experiments.

To confirm SSL11 did indeed localise with gelatinase granules, a gelatinase assay was performed. Porcine gelatin was incorporated into a SDS-PAGE gel so that when stained with coomassie the entire gel would be coloured. In the presence of gelatinase the gelatin degrades to produce a clear band in the gel. Gelatinase activity is observed starting from fraction 8 with stronger activity being observed from fraction 18 correlating with the location of SSL11 (fig. 5.6). SSL11 does not inhibit the activity of gelatinase as there is degradation of gelatin in the fractions. There was no gelatinase in the initial fractions which contain MPO positive azurophilic granules (as determined by a MPO assay – see appendix 7.3.7).
Figure 5.6. Gelatinase assay on neutrophil subcellular fractions demonstrates SSL11 is present in fractions also containing gelatinase

Gelatin gels stained with coomassie showing areas of gelatinase activity (clear). Fraction numbers from the fractionation are shown above the gel. Images are representative of gels run in duplicate for each fractionation assay.
Chapter 5 – Examining internalised SSL11 in myeloid cells

5.2.4. Chemical inhibition of SSL11 entry into myeloid cells

There are several inhibitors of clathrin- and caveolin-dependent endocytosis which are used to determine the entry route of a desired protein (for a comprehensive book see [236]). These include the clathrin-dependent endocytosis inhibitors: hypertonic sucrose, chlorpromazine, phenylarsine oxide and Pitstop 2 [236-239]. Inhibition of endocytosis by hypertonic sucrose is by dispersal of the clathrin lattice at the plasma membrane [236, 237, 239]. Chlorpromazine is a protein kinase C inhibitor which is believed to prevent the adaptor protein 2 (AP2) aiding the formation of the clathrin lattice [227, 236, 240, 241]. Phenylarsine oxide is a tyrosine phosphatase inhibitor which has been shown to inhibit clathrin-dependent endocytosis [236, 237, 240, 242]. Pitstop 2 is a novel compound that has been shown to bind the clathrin heavy chain and block endocytosis [238]. Caveolin-dependent endocytosis can be inhibited by disrupting the lipid organisation with the sterol-binding compound filipin [227, 236, 243]. All these inhibitors, despite being widely used in the literature, have non-specific effects and can interfere with alternative endocytic pathways [236, 241]. Pitstop 2 which was the only specific inhibitor of clathrin has recently been implicated in being able to inhibit clathrin-independent endocytosis [244]. However, despite these flaws, these are the only available inhibitors which are routinely used to demonstrate endocytic pathways. All these inhibitors were therefore used so that collectively, they could provide insight into the mechanism of entry by SSL11US6610.

5.2.4.1. Inhibiting entry of SSL11 into neutrophils

All the inhibitors of clathrin-dependent endocytosis prevented entry of SSL11US6610 into neutrophils (fig. 5.7). Sucrose had to be quickly washed off the cells with PBS, immediately before viewing as the refraction of light in sucrose is not suitable for the objective used. As SSL11 internalises rapidly, the cells could not be thoroughly washed, so trace amounts of sucrose reduced the resolution for this sample. Filipin, the inhibitor of caveolin-dependent endocytosis, did not prevent endocytosis of SSL11 (fig. 5.7). All of these inhibitors (with the exception of chlorpromazine) are insoluble in water, so DMSO was used as a solvent. DMSO is toxic to cells at higher concentrations, so a DMSO control was required. There were no observed cytotoxic effects at the concentrations of DMSO used and SSL11 internalisation was not inhibited.
SSL11 Alexa Fluor 488 (0.25μM - green) with neutrophils in the presence of chemical inhibitors. PhaO is phenylarsine oxide, Suc is sucrose, Chlorp is chlorpromazine, Pit is Pitstop and Fil is filipin. Images represent approximate centre of cell based on z series. Samples representative of four repeat experiments.
5.2.4.2. Inhibiting entry of SSL11 into macrophages

SSL11<sub>US6610</sub> requires active transport into primary macrophages, as shown by the lack of internalised SSL11 when added to cells at 4°C (fig. 5.8). As in figure 3.16, the staining was more fibrous when compared to the punctate staining in neutrophils. Individual vesicles can also be seen containing Alexa fluor 647 labelled SSL11. Internalisation was prevented by the treatment of macrophages with clathrin-dependent endocytosis inhibitors phenylarsine oxide and chlorpromazine (fig. 5.9). Internalisation is not prevented by the addition of filipin, the caveolin-dependent endocytosis inhibitor (fig. 5.9).

![Figure 5.8. Live cell imaging of primary macrophages incubated with SSL11 demonstrates that internalisation is an active process](image)

Primary macrophages incubated with 0.25μM Alexa Fluor 647 labelled SSL11 (red) at room temperature (RT) or 4°C (4). Images represent approximate centre of cell based on z series, experiment done in duplicate.
Chapter 5 – Examining internalised SSL11 in myeloid cells

5.2.5. Neutrophil aggregation with chemical inhibition of SSL11 internalisation

Mentioned in 3.2.6.1, aggregation of neutrophils was hypothesised to be a consequence of the SSL11 dimer crosslinking neutrophils. If the action of internalised SSL11 was responsible, this can be examined using the inhibitors of clathrin-dependent endocytosis. Internalisation of SSL11_{US6610} into neutrophils was inhibited by the addition of phenylarsine oxide, chlorpromazine, and Pitstop before adding SSL11_{US6610} to examine aggregation. As controls PBS, DMSO and filipin were used. As expected all samples exhibited aggregation, supporting the hypothesis that surface dimerised SSL11 crosslinks the neutrophils (fig. 5.10). However the samples that had SSL11 internalisation prevented by the clathrin-dependent endocytosis inhibitors were phenotypically different from the controls (fig. 5.10). The aggregates in these samples were less compact with individual cells being identifiable.
Chapter 5 – Examining internalised SSL11 in myeloid cells

Figure 5.10. SSL11 induced neutrophil aggregation is less compact in the presence of clathrin inhibitors

Chemical inhibitors used: phenylarsine oxide (PhaO); chlorpromazine (chlorp); Pitstop and filipin. Scale is 40μm for all images. Images are representative of four experiments.
5.2.6. Observing the interaction of SSL11 with the cytoskeletal network

5.2.6.1. SSL11 trafficking along the microtubule network

As SSL11 is most likely entering cells via clathrin-mediated receptor endocytosis, it would end up in endosomes which are carried along the microtubule network by dynein and kinesin [220]. The microtubule network spans the entire cell originating from the microtubule organising centre (MTOC) and is responsible for providing the scaffold for the trafficking of vesicles within the cell [220]. SSL11 within endosomes running along the microtubules would provide the fibrous appearance observed in live cell staining along with the observation that internalised SSL11 traverses the entire cell. Confocal microscopy was used to view SSL11’s proximity to the microtubule network. Primary macrophages were used owing to the cell size, providing easier resolution of structures in comparison to neutrophils. Initially tubulin tracker green along with SSL11 US6610 labelled with Alexa Fluor 647 was used so that a live time-course could be made. Unfortunately as seen in figure 5.11, the tubulin tracker green was unable to resolve the individual filaments completely. Individual vesicles containing SSL11 could be observed along with the typical strong staining of the membrane.

Figure 5.11. Live confocal imaging of SSL11 in primary macrophages with stained microtubules

SSL11 conjugated to Alexa Fluor 647 (red) in primary macrophages co-stained with tubulin tracker (green). Images are representative of the cell population of experiments done in triplicate.
Instead of tubulin tracker, antibodies that target \( \alpha \)-tubulin were used on fixed samples to view the microtubule network. SSL11 was internalised prior to fixation so that it would be trafficked as in a live cell. SSL11 was found close to the microtubule network, especially from the periphery to the centre of the cell (fig. 5.12a). A digital zoom enabled closer observation showing areas of close association between the microtubule network and SSL11 (fig. 12b). Single staining of SSL11 or the microtubules did not exhibit any overflow in emission signal between the two channels (data not shown). Controls included having anti-SSL10 as a primary antibody or excluding the primary antibody, neither of which displayed any fluorescent signal indicating there is no cross-reactivity of the antibodies used (data not shown).

**Figure 5.12. Confocal imaging of SSL11 associating with microtubules in fixed primary macrophages**

A) SSL11\textsubscript{US6610} conjugated to Alexa Fluor 488 (green) and anti-\( \alpha \)-tubulin (detected with secondary conjugated to Alexa Fluor 594) shown in red in primary macrophages. B) A digital zoom in a different cell allowing closer examination. Arrows indicated close association between SSL11 and the microtubule network. Images are representative of the cell population from experiments done in triplicate.
Chapter 5 – Examining internalised SSL11 in myeloid cells

5.2.6.2. Observing SSL11 and actin

As mentioned previously, SSL11 has been shown to cause actin modifications when added to neutrophils [122]. The effect SSL11 has on neutrophil chemotaxis (4.2.1) may also involve interference with actin dynamics. To examine if SSL11US6610 can directly associate with actin, microscopy was used along with the protein phalloidin which is incorporated into actin microfilaments. Phalloidin in neutrophils stained the entire cell, likely reflecting the highly motile nature of these cells (fig. 5.13a). Similarly, with the exception of the nucleus, SSL11 stained the entire neutrophil when fixed. This made it impossible to say if there were direct interactions between SSL11 and actin. Primary macrophages (which had more defined actin microfilaments) were used as an alternative to neutrophils (fig. 5.13b). SSL11 showed no association with phalloidin and was observed mostly in the typical region proximal to the nucleus. There was no spectral overlap in the single stained control indicating there is not overlapping emission in the double stained samples (data not shown).

Figure 5.13. Confocal imaging of SSL11 and actin microfilaments showing no association

Phalloidin conjugated to Alexa Fluor 488 (green) and SSL11US6610 conjugated to Alexa Fluor 647 (red) staining fixed: A) neutrophils or B) primary macrophages. Images are representative of the cell population from experiments done in triplicate.
5.2.6.3. Observing SSL11 and vimentin

Vimentin could be one of the two bands identified in the same region as the SSL11 dimer from section 3.2.8.1. Vimentin is a monomer which polymerises to form intermediate filaments which are often closely associated with the other cytoskeletal proteins [245]. SSL11$_{US6610}$ associating with vimentin in macrophages was investigated using confocal microscopy (fig. 5.14). There was no association observed between SSL11 and the vimentin intermediate filaments. SSL11 was observed in a perinuclear space regardless of whether it was internalised pre-fixation or post-fixation (data not shown). Controls were similar to those used for visualising tubulin.

Figure 5.14. Confocal imaging of SSL11 and vimentin intermediate filaments showing no association

SSL11$_{US6610}$ conjugated to Alexa Fluor 488 (green) and anti-vimentin (detected with secondary conjugated to Alexa Fluor 594) shown in red in fixed primary macrophages. Images are representative of the cell population for experiments done in duplicate.
5.2.7. Visualising SSL11 and cellular organelles

As SSL11 has been shown to be endocytosed into cells along with being seen close to the microtubule network, it is likely SSL11 is in an endosome and is being carried to organelles within the cell. Endosomes are trafficked to one of three locations: lysosome; Golgi apparatus or can be recycled back to the plasma membrane [220]. If the endosome ends at the Golgi, its contents can be retroactively transported to the ER or it can be sent back to the plasma membrane or lysosome [220]. If like some other toxins, SSL11 could leave the endosome at any stage during this process. For example, anthrax lethal factor escapes the endosome when the pH drops [225]. Ricin, shiga toxins and cholera toxin are trafficked through the Golgi to the ER whereby they escape the ER to the cytosol via the misfolded-protein transporter [231, 246]. For these reasons, it was decided to examine if SSL11 is able to enter these organelles using confocal microscopy. For all experiments single stained samples and secondary only controls confirmed there was no overlapping emissions between probes or antibody cross-reactivity (data not shown).

5.2.7.1. Visualising SSL11 and the late endosome

The mannose-6-phosphate receptor (M6PR) is a receptor that recycles from the Golgi or plasma membrane to the lysosome and has been used as a marker for the late endosome [236]. SSL11US6610 was internalised into macrophages prior to fixation and probing for the M6PR with monoclonal antibody. Figure 5.15a shows SSL11 located in a typical perinuclear structure coinciding with the location of the late endosome. A digital zoom on a region demonstrated that there were endosomes co-labelling with both SSL11 and M6PR (fig. 5.15b).
Examining internalised SSL11 in myeloid cells

Figure 5.15. Confocal imaging of SSL11 inside late endosomes in fixed macrophages

A) SSL11_{US6610} conjugated to Alexa Fluor 488 (green) and anti-M6PR (detected with secondary conjugated to Alexa Fluor 594) shown in red in fixed primary macrophages. B) A digital zoom of the framed area from (A) with dual staining endosomes indicated with arrows. Images are representative of the cell population for experiments done in triplicate.

5.2.7.2. Visualising SSL11 and the Golgi apparatus

NBD C₆-ceramide is a fluorescent lipid probe that allows the detection of the Golgi apparatus in live cells. Dual staining was performed with SSL11_{US6610} to examine if SSL11 was transported to the Golgi apparatus of primary macrophages (fig. 5.16a). While the Golgi appeared in the same perinuclear area as SSL11, there was no observable co-localisation. Unlike previous observations of the perinuclear SSL11 structure, there were void areas in SSL11 staining which coincided with the Golgi stain. It was thought that perhaps the NBD C₆-ceramide emission may be quenching or interfering with SSL11. An antibody directed against giantin, a marker of the Golgi, was used in fixed macrophages instead to alleviate this possibility (fig. 5.16b). As for NBD C₆-ceramide, SSL11 and giantin occupy a similar space proximal to the nucleus. Despite this close association, it was difficult to determine if there was true co-localisation between the giantin and SSL11 fluorescence which occupied a large area.
Chapter 5 – Examining internalised SSL11 in myeloid cells

Figure 5.16. Confocal imaging of SSL11 and the Golgi apparatus

A) Live cell imaging of NBD-C₆ ceramide (green) labelling the Golgi and SSL11 conjugated Alexa Fluor 647 (red) in primary macrophages. B) SSL11 conjugated Alexa Fluor 647 (red) internalised into macrophages prior to fixation and labelling the Golgi with anti-giantin (detected with secondary conjugated to Alexa Fluor 488) which is shown in green. Images are representative of the cell population for experiments done in duplicate.

5.2.7.3. Visualising SSL11 and the endoplasmic reticulum

Live cell imaging of SSL11₁₀₆₆₆₆₁₀ trafficking to the ER in primary macrophages was performed by labelling the cell with ER tracker (fig. 5.17a). The ER tracker however did not resolve any structures in these macrophages and stained the entire cytoplasm. So an antibody targeting calnexin, an ER marker, was used to probe fixed macrophages as performed previously. The staining was widely distributed throughout the cell with now more resolved structures (fig. 5.17b). SSL11 staining was in areas of particularly weak staining for calnexin and so is unlikely to be transported to the ER. An antibody directed to another ER marker, protein disulfide isomerase, detected with a different fluorophore produced a similar staining profile as calnexin (data not shown).
Chapter 5 – Examining internalised SSL11 in myeloid cells

5.2.7.4. Visualising SSL11 and lysosomes

Lysosomal-associated membrane protein 1 (LAMP1) is a marker for detecting lysosomes (and endosomes being trafficked to the lysosome) within the cell, along with recycling endosomes that are shuttled through the lysosome. An antibody was used to detect these organelles within fixed macrophages along with SSL11US6610 that had been internalised prior to fixation (fig. 5.18). The LAMP1 marker was in vesicles throughout the cell, with dense staining being observed in a similar region to that of SSL11. It is difficult to say if there was true co-localisation as the SSL11 staining was more dispersed.

Figure 5.17. Confocal imaging of SSL11 and the Endoplasmic reticulum

A) Live cell imaging of ER tracker (green) and SSL11 conjugated Alexa Fluor 647 (red) in primary macrophages. B) SSL11 conjugated Alexa Fluor 647 (red) internalised into macrophages prior to fixation and labelling the ER with anti-calnexin (detected with secondary conjugated to Alexa Fluor 488) which is shown in green. Images are representative of the cell population for experiments done in duplicate.
Chapter 5 – Examining internalised SSL11 in myeloid cells

5.2.8. Transmission Electron Microscopy of SSL11 in neutrophils

Transmission Electron Microscopy (TEM) was used to try and visualise SSL11\textsubscript{US6610} within organelles in the cells by using monoclonal anti-SSL11 and a gold conjugated secondary antibody to detect SSL11. Unfortunately in order to preserve the antigenicity of SSL11, milder fixation was required which did not preserve the cellular ultrastructure. However, as seen in figure 5.19, the nucleus was still distinct and contained a collection of gold labelled SSL11. In the control that received no SSL11 (fig. 5.19i) or no primary antibody (fig. 5.19ii) there was non-specific binding with some gold labelling in the nucleus. This did not compare to the frequency of gold staining in samples that received SSL11 and both antibodies (fig. 5.19iii-v). Staining of SSL11 was quite often in clusters. Occasionally SSL11 was found in what would likely be the nuclear membrane which may represent an entry point into the nucleus (fig. 5.19iv green arrow).

Figure 5.18. Confocal imaging of SSL11 and lysosomes

SSL11 conjugated Alexa Fluor 647 (red) internalised into macrophages prior to fixation and labelling the ER with anti-LAMP1 (detected with secondary conjugated to Alexa Fluor 488) which is shown in green. Images are representative of the cell population for experiments done in duplicate.
Figure 5.19. Transmission electron microscopy of SSL11 in neutrophils

i) Neutrophils without SSL11; ii) neutrophils with SSL11 and secondary; iii and iv) Neutrophils with SSL11 and both primary and secondary antibodies in the cytoplasm and closer to the nucleus. Gold particles are shown with red arrows in the nucleus (N) or cell (C). The blue arrow indicates a gold particle in what is likely the nuclear membrane. Images are representative of the cell population for experiments done in duplicate.
Chapter 5 – Examining internalised SSL11 in myeloid cells

5.3. Discussion

All alleles of SSL11 were able to internalise into both neutrophils and macrophages despite differences in binding affinities for sLe\(^\text{\textendash}\). The SSL11 alleles from Newman and US6610 exhibited intense membrane staining unlike the alleles from JSNZ and GL10. This would indicate that either it is the monomer involved in internalisation, or the excess SSL11 dimer is not dissociating off the cell. It is hypothesised that the latter is true as the dimer produced an altered dissociation curve, when compared to the monomer, whereby it was not released in the measured time (fig. 3.8). It is also believed, as mentioned in chapter 3, that SSL11 dimerisation is an important process in inducing its internalisation. The monomer was still internalised into neutrophils which is believed to be a consequence of SSL11 concentrating on the cell surface to enable dimerisation. Internalised SSL11 did not display any cleavage products, which was supported by no observable cleavage products when using Sulfo-SBED conjugated SSL11. This does not exclude any modifications achieved by alternative means.

SSL11 was found in gelatinase and possibly secretory granules. It is not currently known which granule in particular, and this would need to be resolved by ELISA for specific target antigens. Granules are formed during the maturation of neutrophils allowing mature neutrophils to respond quickly to infection [247]. It is possible SSL11 is associated with these granules following endosome fusion with the granule. One notable feature of gelatinase and secretory granules is that they are both rich in the receptor Mac-1 [235]. SSL11 bound to Mac-1 could accommodate for this finding as SSL11 would then be able to bind these receptors on the granule membrane. It is not known if Mac-1 could be recycled directly to the granules.

Using chemical inhibitors of endocytosis, SSL11 was shown to internalise into neutrophils and macrophages via clathrin-dependent endocytosis rather than caveolin-dependent endocytosis. While these inhibitors are not completely reliable, they are currently the best indicators of endocytosis without knocking out the clathrin gene (which produces other adverse effects on the cell). Phagocytosis cannot be excluded which is also regulated by Mac-1 [20]. Cytochalasin D, which interferes with actin and is used to inhibit phagocytosis, was also trialled but had adverse effects on the neutrophils making them look apoptotic (data not shown) [248]. For this reason it could not be deduced what contribution phagocytosis has on SSL11 internalisation. When SSL11 internalisation was inhibited with the clathrin-dependent endocytosis inhibitors, there was no disruption to the formation of neutrophil aggregates.
However the aggregates were less dense indicating that the internalisation of SSL11 may contribute to the proximity of cells during aggregation. As mentioned previously, aggregation is achieved by Mac-1 adhesions stabilising interactions between L-selectin and sialylated proteins [28, 183]. If SSL11 was endocytosed with Mac-1, perhaps this triggers a more stable interaction of L-selectin with its glycoprotein. Endocytosis is linked to the cytoskeleton, in particular actin [249]. Endocytosis of Mac-1 induced by SSL11 may also alter actin microfilaments which would alter the cell shape and allow closer interactions.

Cellular staining in primary macrophages revealed SSL11 in vesicles, supporting the observation that SSL11 is endocytosed. Endocytosis was also supported by the observation of SSL11 in close proximity to microtubules. It is likely that SSL11 in endosomes would be transported along the microtubule network by dynein. This was also supported by the finding that SSL11 could be seen along with M6PR in late endosomes. Unfortunately no clear co-localisation was seen with Rab5 or Rab11 which are responsible for directing endosomes during endocytosis and recycling to the Golgi respectively (data not shown) [250]. There was no association between SSL11 and actin microfilaments or vimentin intermediate filaments. SSL11 was always abundantly found in a region close to the nucleus. This perinuclear staining was observed regardless of whether SSL11 was added prior to or post fixation and permeabilisation. This indicates one of two possibilities. The first possibility is that SSL11 is able to escape to the cytoplasm after endocytosis, which mimics SSL11 directly entering the cell cytoplasm through a permeable membrane. This may explain the observed dispersed SSL11 staining and would allow access to the nucleus. The second is that SSL11 is shuttling with a membrane receptor which it can bind to within the cell organelle membranes if added when the plasma membrane is permeabilised. This supports the idea that SSL11 is inducing endocytosis of a receptor such as Mac-1 which is abundant within the cell and on the cell membrane.

Unfortunately no obvious co-localisation was seen between SSL11 and markers for the Golgi apparatus, endoplasmic reticulum or lysosome. Although it is possible that SSL11 is transported to the Golgi or lysosome as they occupy similar regions within the cell. SSL11 staining is quite disperse around this perinuclear region which may also indicate the possibility that it can enter the cytoplasm. More direct methods will be required to determine where SSL11 is transported to.
Interestingly SSL11 was observed in the nucleus under TEM. This is in contrast to the immunofluorescence which never showed SSL11 in the nucleus. Perhaps the fixation and permeabilisation process did not preserve the nuclear components in these samples. It must be noted that there was non-specific binding for the antibodies in the TEM samples, which may be enhanced by SSL11 and could be producing false positives. However the increased frequency of gold label in the SSL11 loaded sample warrants investigation into whether SSL11 is able to enter the nucleus.

In summary, SSL11 is endocytosed into myeloid cells via clathrin-dependent endocytosis. SSL11 is then trafficked along the microtubule network to an unknown structure. It is most likely that SSL11 is targeted to the Golgi apparatus or lysosome.
Chapter 6. Discussion

6.1. Introduction

*S. aureus* produces an estimated 200 to 300 virulence factors [90]. With such a vast arsenal, it is not surprising that *S. aureus* is such a successful pathogen, exhibiting the ability to colonise almost any tissue within the human body. Such a trait is dependent on the capacity to avoid immune surveillance. As the SSLs contribute to the evasion of the immune defences, they are clearly important factors to the survival of the bacteria within the host. This importance has been demonstrated further by the observation that membrane damage induced by hemin toxicity and gramicidin, as well as the presence of active neutrophils, up-regulate the expression of the *ssl* genes [129, 132, 133]. The expression of *ssl* genes, like *spa* (protein A) and *fnbA/fnbB* (FnbAB), is controlled by Rot and SaeRS regulators unlike other virulence factors such as the leukocidins, haemolysins and superantigens which are controlled by Agr [61, 132]. From this duality of regulators, it is hypothesised that the SSLs aid the bacteria in keeping hidden from the immune surveillance, once colonisation has been established, whereas the Agr regulated virulence factors perturb immune responses during bacterial invasion. This hypothesis is supported by observation that SSLs have been shown to bind sensory molecules such as immunoglobulins and toll-like receptors as well as components of chemotaxis including complement proteins and cellular adhesins such as PSGL-1 [118, 135, 140, 142, 150, 153, 158]. Past research on SSL11 has shown it capable of binding and internalising into neutrophils. The aim of this research was to further characterise the interaction and activity of SSL11 with myeloid cells as well as the process of internalisation, so as to further understand the benefit it provides to this immune evasive phenotype.
6.2. Analysis of conservation of the SSL11 sequence

SSL7 has demonstrated, that despite being a small molecule, it is able to bind both IgA and C5 [135, 140]. It is likely that SSL10 exhibits a similar characteristic in binding IgG and a component of the complement or coagulation cascade [142, 143]. Such a feat is achieved due to the conserved nature of the SSL structure, which employs two domains that are known to bind many molecules including oligonucleotides, oligosaccharides and proteins [65, 105, 113]. A comparison of all sequences of SSL11 was performed to examine if another site in the structure was conserved which would highlight a possible binding site distinct to the carbohydrate binding site. However, conservation was only identified around the carbohydrate site, highlighting its importance. This is reflective of the low sequence identity of the SSL11 alleles where identity is mostly conserved to the β-grasp domain. This is unlike other SSLs such as SSL3, SSL4, SSL9 and SSL10 which display much more conservation among the different strains [119, 142, 143, 146]. It is therefore believed that SSL11s primary function is related to the interaction with the sialylated molecule.

6.3. Dimerisation of SSL11

SSL11 had been observed to form a dimer when crystallised in a similar manner to that of SSL5 [113, 122]. The dimer, which spontaneously formed in solution, was separated by size exclusion and exhibited different properties to the monomer. However, not all alleles formed dimers in solution at the concentrations tested. It is expected they would form dimers at higher concentrations. The dimer had only a slight increase in affinity when compared to the monomer for both sLacNAc and sLe^a. It is likely that the published affinity for SSL11 binding sLe^a is the dimer, based on the sensogram and the high concentration of the SSL11 used [118]. The dissociation of the dimer from the carbohydrate was greatly diminished and insinuates that dimerised SSL11 would remain attached to the cell for considerably longer periods. It is likely that this prolonged attachment is what contributes to SSL11-induced aggregation of myeloid cells as it was the dimer that cross-linked the neutrophils in a manner imitating the homotypic aggregation caused by L-selectin binding sialylated glycoproteins. Currently, the only known differences between the SSL11 alleles are that JSNZ and GL10 do not readily form dimers in solution at the concentrations tested. This supports the claim that the SSL11 dimer cross-links neutrophils as these two alleles did not aggregate neutrophils until higher concentrations were applied. This also collectively demonstrates that dimerization is independent of SSL11 binding carbohydrate as the monomer of JSNZ had the
highest affinity of all SSL11 alleles for sLe\(^x\). This is also supported by the ability of the carbohydrate binding mutant of US6610 still being able to dimerise.

From experimentation performed I hypothesise that the importance of the dimer is that it induces the endocytosis of SSL11 into neutrophils. Sialylated receptors are concentrated into rafts or microdomains to provide them proximity to their appropriate signalling molecules allowing them to function \([40, 58]\). Activation of the receptor is often achieved via conformational changes and receptor oligomerisation following ligand binding. If SSL11 concentrated in a pit, allowing for dimerization, this would non-specifically aggregate the glycoreceptors that SSL11 bound to, possibly resulting in the activation of their pathways which could include endocytosis. It is believed that this is the mechanism used by most AB toxins that bind cell receptors, as oligomerisation of the toxin is required for endocytosis \([221, 225, 242, 246, 251, 252]\). A clearer understanding would be obtained once the identity of the receptor is known. However, this hypothesis is supported by the observed concentrated domains of SSL11 on the surface of macrophages with the internalised SSL11 originating from these domains. Similarly the higher density of SSL11 at the uropod, where it is known receptors concentrate also provides support for this hypothesis.

It would be interesting to speculate on the ability of SSL11 to dimerise with the other SSLs. This could bring together differing molecules and pathways allowing for more complex immune evasive strategies. It is a possibility as all the SSLs share the same structure. However there are differences in the curvature, length and adjacent loops of the \(\beta 7\) strand which may perturb the possibility of forming heterodimers. As each \(ssl\) gene has its own promoter, this insinuates that each SSL protein may perform individually.

It is difficult to demonstrate a direct link between the SSL11 dimer and its function as the crystal structure explains that the dimer is mainly formed by hydrogen bonds between primary amines and carboxyl groups (rather than their sidechains) of the flat \(\beta 7\) strand surface \([122]\). This means point mutations would be ineffective and removal of the strand would most likely be detrimental to the structure. Perhaps the introduction of a cysteine into the \(\beta 7\) strand would allow for targeted protein cross-linking of SSL11 with the carbohydrate binding mutant which could dimerise, but only bind one receptor and so could not aggregate the receptors.
Chapter 6 – Discussion

6.4. SSL11 binding sialylated receptors

Sialylated molecules are an important component of immune recognition and regulation meaning they are a common conjugate of immune receptors [53, 58]. SSL11 had been shown to bind the core sialyllactosamine with the α2-3 linkage between the sialic acid and galactose being the most important determinant of binding affinity [118]. However beyond this core stoichiometry, there appears to be no selectivity as seen by SSL11 having the same affinity for both sLacNAc and sLeα. This explains why SSL11 does not compete with itself or titrate at the concentrations used in the cell binding studies, as it will promiscuously bind any correctly sialylated molecule.

6.4.1. Gangliosides

It has not been investigated if SSL11 is able to bind gangliosides containing the Neu5Aca2-3Gal moiety, of which GM1 and GM3 are important to the immune system [253]. GM3 (Neu5Aca2-3Galβ1-4Glc1-1Cer) has a glucose instead of N-acetylglucosamine as tested in sialyllactosamine, whereas GM1 (Galβ1-3GalNAcβ1-4[Neu5Aca2-3]Galβ1-4Glc1-1Cer) also has an extra galactose and N-acetylgalactosamine. Cholera toxin is an example of a toxin that binds GM1 and utilises its association in concentrated lipid rafts to direct the internalisation of the toxin into the intestinal epithelia [226, 246]. Similarly SabA produced by Helicobacter pylori also binds sialylated carbohydrates such as sLeα as well as gangliosides which are present in neutrophils [254, 255]. If SSL11 could bind these gangliosides, its cellular tropism would be broad as gangliosides are ubiquitous and are especially important in the nervous system [256]. GM1 is a component of the nuclear membrane and may account for the close association between SSL11 and the nucleus [257]. Gangliosides are involved in many processes related to receptor reorganisation, recognition and signalling which could be perturbed by SSL11 binding. One such important example is that E-selectin uses gangliosides on neutrophils as receptors to allow adhesion of the neutrophils to endothelia [26, 256].
6.5. Is there a specific receptor for SSL11?

Despite the promiscuous binding exhibited by SSL11 towards many sialylated glycoproteins, it is hypothesised that there is a specific receptor that SSL11 targets. It is believed that it is this receptor that allows the internalisation of SSL11 as not all SSL11 is internalised when viewed by microscopy. Once the specific receptor is saturated, SSL11 can then bind more sialylated receptors resulting in the observed promiscuity. Excess SSL11 on the surface would then contribute to the aggregation of cells via dimerization, crosslinking the cells. This hypothesis is supported by the finding that SSL4, a carbohydrate binder, competes with itself when applied at lower concentrations which suggests there is a preferred receptor [119]. SSL4 forms more hydrogen bonds with fucose and so exhibits a higher affinity for sLe$^x$ when compared to sLacNAc [119]. Such a difference allows for the competition to be observed for SSL4, unlike SSL11.

6.5.1. Macrophage – 1 antigen

The most likely candidate receptor for SSL11 is Mac-1, a heterodimer formed between integrin $\alpha$M (CD11b) and $\beta$2 (CD18). SSL11 has shown to directly bind CD11b as seen in granulocyte pulldowns [122]. Both integrin domains contain glycosylations with terminal sLe$^x$ which would allow SSL11 to bind [258]. Aggregation of Mac-1 is a necessary step involved in its activity and endocytosis, supporting the notion that dimerization of SSL11 on the surface drives its internalisation [180, 181]. Endocytosis of Mac-1 is also associated with actin signalling and may account for the observed changes in actin seen in fMLF activated cells [25, 259]. These changes were only observed in activated cells and not resting cells. This may relate to the fact that in an activated neutrophil, degranulation increases the amount of Mac-1 found on the cell surface which then migrates to the uropod [19, 20, 31, 235]. SSL11 was found to also be concentrated more at the uropod in activated neutrophils which may reflect SSL11 migrating along with Mac-1 or binding directly at the uropod. SSL11 was also found associated with gelatinase and possibly secretory granules which are the source of stored Mac-1 in unactivated cells. If SSL11 is trafficked along with Mac-1 from the plasma membrane to the lysosome, this would result in diminished surface Mac-1 along with the destruction of the trafficked Mac-1 which would be beneficial to the bacteria as the innate cells could not migrate or perform phagocytosis effectively.
Mac-1 is also involved in neutrophil homotypic aggregation, along with L-selectin [28]. Perhaps endocytosis of Mac-1 with SSL11 promotes a cellular change that allows the excess SSL11 to form tighter aggregates. Inhibition of this process with chemical inhibitors would then abrogate this process. Antibodies directed against CD11b have been shown to inhibit neutrophil aggregation which may involve a similar process (as L-selectin is not blocked) [260]. The major argument against the possibility of Mac-1 being the specific receptor is the reduced internalisation into THP-1 cells. THP-1 cells express Mac-1 so this observation cannot be currently explained. Perhaps alternative glycosylation, abundance or other factors may be different in this cell line.

Currently, no increases in SSL11 binding activated neutrophils have been observed. Similarly competition between SSL11 and anti-CD11b has not been observed. However this may reflect the difficulty in identifying binding of a specific receptor amongst binding of other receptors. The adenylate cyclase toxin from *B. pertussis* is an example of a toxin that binds Mac-1 which then scaffolds the toxin to directly enter the membrane while also promoting the internalisation of the integrin [261].

### 6.6. Parallels to the AB family of toxins

The AB family of toxins represent intracellularly active toxins that enter cells without the use of a secretion system [221]. They are named based on containing an: A domain which has the functional activity; and a B domain which binds the cell and shuttles the A domain to its target [221]. SSL11 has several similarities to the B domains of AB toxins in that it binds the cells, is internalised and is trafficked through the cell. Despite efforts shown here, it has not yet been fully determined where in the cell SSL11 is targeted to. The most likely target organelles are the Golgi or lysosome, while the cytoplasm or nucleus are also possible but less likely. It is interesting to consider, that SSL11 trafficking along with its bound receptor could lead to its degradation in the lysosome. SSL11 has not exhibited any cytotoxic effects within the cell. Perhaps SSL11 is similar to the B domain in that it shuttles molecules into the cell. The *ssl11* gene is expressed with the next gene directly downstream, which is present in all strains containing *ssl11*[128]. The product of this gene has no known function and has a predicted secretion cleavage site along with a predicted YSIRK cell wall anchor sequence. Little is known about the YSIRK motif, but it has been shown to be important in polarising receptors of dividing *Streptococcus pyogenes* and *S. aureus* [262]. It may be possible that SSL11 interacts with this hypothetical protein to enact its function, as bacteria often cluster
functionally related genes. If SSL11 was able to bind *S. aureus* either directly, or through the hypothetical protein, it is possible that SSL11 could aid in the endocytosis of the bacteria. Such a process would benefit the survival of bacteria within the neutrophils as they have not entered via the phagocytic pathways. This is supported by a study whereby a mutation of *ssl11* and the downstream gene resulted in a 1.5 log attenuation in virulence in a kidney abscess infection model [263]. Bacteria containing a mutation of the SaeR/S regulatory system that regulate the expression of *ssl* genes was shown to have reduced survivability within neutrophils and may complement this hypothesis [264]. Internalisation of *Mycobacterium tuberculosis*, *Porphyromonas gingivalis*, *Francisella tularensis* and *B. anthracis* spores via Mac-1 endocytosis have been shown to aid the intracellular lifecycle of these bacteria [265-268].

### 6.7. Future directions

#### 6.7.1. Characterisation of SSL11 interacting with Mac-1

As mentioned, competition assays are difficult with SSL11. However, if SSL11 is endocytosed along with Mac-1 competition may be possible with the anti-CD11b antibodies. Similarly a decrease in the neutrophil surface CD11b/CD18 would be expected following SSL11 treatment, which could be detected with anti-CD11b antibodies. It would be interesting to examine if the anti-CD11b also inhibited SSL11 induced neutrophil aggregation as it does Mac-1 homotypic aggregation. Optimisation of immunofluorescence would also allow the examination of any co-localisation between SSL11 and Mac-1. Fluorescent labelling of Mac-1 may also allow real time analysis of its trafficking. Removal of Mac-1 from a cell line that expresses it, and is permissive to SSL11 internalisation, would provide conclusive evidence for its role in the internalisation process.

#### 6.7.2. Examine SSL11 intracellular trafficking

Unlike many bacterial toxins, SSL11 does not appear to be cytotoxic. This means cell death cannot be used as an endpoint to determine if conventional trafficking inhibitors such as Bafilomycin A (inhibits acidification of endosome) or Brefeldin A (arrests Golgi trafficking) have been effective. It may be possible to see if these inhibitors prevent SSL11 from migrating to the conventional structures as seen by microscopy. A direct approach however would be more informative. Subcellular fractionation at much higher speeds would allow the
Chapter 6 – Discussion

separation of organelles. This could be coupled to mass spectrometry to achieve greater sensitivity while examining cellular trafficking [218]. Coupling to mass spectrometry would also allow further characterisation of how SSL11 is altering actin and the lysosomal trafficking regulator.

6.7.3. SSL11 interacting with the downstream hypothetical protein

There has been no evidence for SSL11 interacting with the soluble hypothetical protein when observed via ELISA or by confocal microscopy (data not shown). However this does not accommodate the possibility of the hypothetical protein being found on the S. aureus cell wall. Antibodies directed against this protein would allow the examination, by Western blot, of this possibility. If the hypothetical protein was found attached to the staphylococcal cell wall, further characterisation of its possible interaction with SSL11 could be performed.

6.7.4. In vivo assays

With the observation that SSL11 inhibits the migration of neutrophils as well as blocks P-selectin mediated rolling, it would be beneficial to identify if this is true in vivo. SSL11 has been shown to bind mouse neutrophils (communication with Dr. F. Radcliff). Inflammatory models where neutrophil infiltration predominates would provide insight into the in vivo capabilities of SSL11. The ssl11 gene has been cloned into a vector that allows abundant expression by L. lactis. This knock-in mutation, in similar inflammatory and infection models, would allow the examination of SSL11 in isolation without compounding results from other potential S. aureus virulence factors.

6.8. Concluding remarks

SSL11 represents a complex virulence factor that, due to its binding a ubiquitous carbohydrate, has the potential to perform multiple functions. Data presented in this thesis shows how SSL11 inhibits neutrophil recruitment and narrows down the possible target receptor and intracellular locations. Further characterisation of these properties of SSL11 will enable the identification of the activity of SSL11. Such knowledge will allow for the improved understanding of how SSL11 (and most likely the other carbohydrate-binding SSLs) aid S. aureus in remaining undetectable to the immune system.
7.1. SSL11 Sequence Alignment

Mu50  
---STLVRQQQATQLDSEYYSGKYGFGFELTVNVTGYGYNKVTIFD---NSQIDIVTLGTNEK
71193  
---STLVRQQQATQLDSEYYSGKYGFGFELTVNVTGYGYNKVTIFD---NSQIDIVTLGTNEK
RF122  
DENSSRLAATSKDWTQQLKYYSGTYGNNGFQNSTGVRKMNNGTD---GQLNVNIVLLSDDK
MW2  
-IEQSLRSTVDKQDQLKMYSGTYGNNGFQNSTGVRKMNNGTD---GQLNVNIVLLSDDK
COL  
---STLVRQQQATQLDSEYYNRFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
C1GC345d  
---STLVRQQQATQLDSEYYNRFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
GL10  
---STLVRQQQATQLDSEYYNRFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
US6610  
---STLVRQQQATQLDSEYYNRFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
ED133  
---STLVRQQQATQLDSEYYNRFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
HO50960412  
---STLVRQQQATQLDSEYYNRFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
SAO11  
KSDSSLTIAINNDTKKLDYTDYGFSDFYLYNKLYGREGNATIFP---NSQIDIVTLGTNEK
1189-87  
DEASSRLTSVDQDNLTTQYGTASDNLGSLGGKYGGEDYKVTIFP---NSQIDIVTLGTNEK
JKD6159  
-IEQSLRSTVDKQDQLKMYSGTYGNNGFQNSTGVRKMNNGTD---GQLNVNIVLLSDDK
MRS4252  
-IEQSLRSTVDKQDQLKMYSGTYGNNGFQNSTGVRKMNNGTD---GQLNVNIVLLSDDK
Newhould  
-IEQSLRSTVDKQDQLKMYSGTYGNNGFQNSTGVRKMNNGTD---GQLNVNIVLLSDDK
M013  
-IEQSLRSTVDKQDQLKMYSGTYGNNGFQNSTGVRKMNNGTD---GQLNVNIVLLSDDK
SHHR1132  
-IEQSLRSTVDKQDQLKMYSGTYGNNGFQNSTGVRKMNNGTD---GQLNVNIVLLSDDK
S0385  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
Newman  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
N315  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
JSNZ2  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
JJH  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
JJH9  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
USA300_FPR3757  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
USA300_TCH1516  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
NCTC8325  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
MSA4746  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
ED98  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
04-02981  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
ECT_R2  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
VC40  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
JKD6008  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
TQ103  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
TW20  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
TC60  
---STLVRQQQATQLDSEYYNFNFFETQNSQYEEKGRTVFTF---NYLIDIVTLGTNEK
LQA251  
KSDSSLTIAINNDTKKLDYTDYGFSDFYLYNKLYGREGNATIFP---NSQIDIVTLGTNEK

* * : : : . * * . * * . : * : * : 

Mu50  
LTVKDD--DEVSNVQVFFVRESGSKDATTSIIGITKTNGTTQKTVQDQVNLSDGKGQ
71193  
ERFPNS--DSYGLVQVFFVRESGQADNNSSIGITKTNKNDYDVVNVFNLGIEFTPGHN
RF122  
ERFKDD--EDYGELOVFFVRESGKHAADNISIGITKTNNQDKVDQVNLNSTSKGQ
MW2  
ERFKDD--EDYGELOVFFVRESGKHAADNISIGITKTNNQDKVDQVNLNSTSKGQ
COL  
QNF--Q-EDISNFDVFFVRESNRSGTASIGITKTNQSNYIDVVDKVIINIITKNDSV
C1GC345d  
QNF--Q-EDISNFDVFFVRESNRSGTASIGITKTNQSNYIDVVDKVIINIITKNDSV
GL10  
LEFNNFD--EIEHVDVVFVRESNRSGITSNQGDNQDDVFVSDKVQNDNQNDV
US6610  
DJKGDD--NNENLVDVFFVRESGRQDDNISSIGITKTNRQHTIDTVQVNLVLSKSTQGH
ED133  
LEFNNFD--EIEHVDVVFVRESNRSGITSNQGDNQDDVFVSDKVQNDNQNDV
HO50960412  
LEFNNFD--EIEHVDVVFVRESNRSGITSNQGDNQDDVFVSDKVQNDNQNDV
SAO11  
KKFEDG--NQIGVQVFFVRESGRQATDIISIGITKTNQNYDVVNVFNLGIEFTPGHN
1189-87  
DDVQSN--DVQSNVQVFFVRESQGKAMDNNISIGITKTNQNYDVVNVFNLGIEFTPGHN
JKD6159  
ERFDDG--KDNENLVDVFFVRESGRQADNNSSIGITKTNRQHTIDTVQVNLVLSKSTQGH
MRS4252  
ERFDDG--KDNENLVDVFFVRESGRQADNNSSIGITKTNRQHTIDTVQVNLVLSKSTQGH
Newhould  
ERFDDG--KDNENLVDVFFVRESGRQADNNSSIGITKTNRQHTIDTVQVNLVLSKSTQGH
M013  
ERFDDG--KDNENLVDVFFVRESGRQADNNSSIGITKTNRQHTIDTVQVNLVLSKSTQGH
SHHR1132  
LEFND--EIEHVDVVFVRESNRSGITSNQGDNQDDVFVSDKVQNDNQNDV
S0385  
ERFNS--DSYGLVQVFFVRESGRQADNNSSIGITKTNRQHTIDTVQVNLVLSKSTQGH
Newman  
QNF--Q-EDISNFDVFFVRESNRSGTASIGITKTNQSNYIDVVDKVIINIITKNDSV
N315  
LTVKDD--DEVSNVQVFFVRESGSKAATTISIGITKTNGTTQKTVQDQVNLSDGKGQ
JSNZ2  
ERFNS--DSYGLVQVFFVRESGRQADNNSSIGITKTNRQHTIDTVQVNLVLSKSTQGH
JJH  
LTVKDD--DEVSNVQVFFVRESGSKAATTISIGITKTNGTTQKTVQDQVNLVLSKSTQGH
JJH9  
LTVKDD--DEVSNVQVFFVRESGSKAATTISIGITKTNGTTQKTVQDQVNLVLSKSTQGH
Mu3  
LTVKDD--DEVSNVQVFFVRESGSKAATTISIGITKTNGTTQKTVQDQVNLVLSKSTQGH
USA300_FPR3757  
QNF--Q-EDISNFDVFFVRESNRSGTASIGITKTNQSNYIDVVDKVIINIITKNDSV
USA300_TCH1516  
QNF--Q-EDISNFDVFFVRESNRSGTASIGITKTNQSNYIDVVDKVIINIITKNDSV
NCTC8325  
QNF--Q-EDISNFDVFFVRESNRSGTASIGITKTNQSNYIDVVDKVIINIITKNDSV
7.2. Plasmids

pET32a3C was modified from pET32a (Novagen) by Dr. Thomas Proft
## 7.3. Mass Spectrometry results

### 7.3.1. SSL11

**Mascot Search Results**

<table>
<thead>
<tr>
<th>Taxonomy</th>
<th>Other Firmicutes (2427066 sequences)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timestamp</td>
<td>26 Apr 2012 at 02:30:53 GMT</td>
</tr>
<tr>
<td>Protein</td>
<td>gi</td>
</tr>
<tr>
<td>gi</td>
<td>30682 8718</td>
</tr>
</tbody>
</table>

**Mascot Score Histogram**

Ions score is $-10\log(P)$, where $P$ is the probability that the observed match is a random event. Individual ions scores $> 49$ indicate identity or extensive homology ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

### Peptide Summary Report

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Charge</th>
<th>Mass (Mr(calc))</th>
<th>Score</th>
<th>Matches</th>
<th>Sequences</th>
<th>emPAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>K.NGDFYTFELNK.K</td>
<td>+1</td>
<td>22638.000</td>
<td>307</td>
<td>10(3)</td>
<td>8(3)</td>
<td>2.02</td>
</tr>
<tr>
<td>K.DGNNENLDVFVVR.E</td>
<td>+1</td>
<td>1807.970</td>
<td>46</td>
<td>92</td>
<td>51</td>
<td>2.02</td>
</tr>
<tr>
<td>K.R.TQHIDTVQNV</td>
<td>+1</td>
<td>1808.960</td>
<td>32</td>
<td>92</td>
<td>61</td>
<td>2.02</td>
</tr>
<tr>
<td>K.R.TQHIDTVQNNLLVSK.S + Deamidated (NQ)</td>
<td>+1</td>
<td>1808.960</td>
<td>46</td>
<td>92</td>
<td>61</td>
<td>2.02</td>
</tr>
<tr>
<td>K.R.TQHIDTVQNNLLVSK.S</td>
<td>+1</td>
<td>1730.870</td>
<td>47</td>
<td>92</td>
<td>61</td>
<td>2.02</td>
</tr>
<tr>
<td>K.R.SQATQDLSEYYNRPYFDLR.N</td>
<td>+1</td>
<td>2365.080</td>
<td>36</td>
<td>92</td>
<td>41</td>
<td>2.02</td>
</tr>
<tr>
<td>K.R.EGNTVTFINHYQQTDVK.L</td>
<td>+1</td>
<td>1992.920</td>
<td>51</td>
<td>92</td>
<td>41</td>
<td>2.02</td>
</tr>
<tr>
<td>K.R.EGNTVTFINHYQQTDVK.L + Deamidated (NQ)</td>
<td>+1</td>
<td>1992.920</td>
<td>51</td>
<td>92</td>
<td>41</td>
<td>2.02</td>
</tr>
<tr>
<td>K.R.STGQHTTSVTSTNSYIYKEEISLK.E</td>
<td>+1</td>
<td>2673.270</td>
<td>32</td>
<td>92</td>
<td>61</td>
<td>2.02</td>
</tr>
</tbody>
</table>

1. **Error tolerant**

Check to include this hit in error tolerant search
7.3.2. Vimentin

**Taxonomy** : Homo sapiens (human) (245558 sequences)

**Timestamp** : 26 Apr 2012 at 02:30:27 GMT

**Protein hits** :

- gi|62414289 vimentin [Homo sapiens]
- gi|1218 keratin 1 [Homo sapiens]
- gi|476 cytokeratin 9 [Homo sapiens]
- gi|97635 beta actin variant [Homo sapiens]
- gi|439935 Chain A, Crystal Structure Of Vimentin (Fragment 144-251) From Homo Sapiens, Northeast Structural Genomics Consortium Target Hr4796b keratin 10 [Homo sapiens]
- gi|283 unnamed protein product [Homo sapiens]
- gi|685 alpha-actin [Homo sapiens]
- gi|403 keratin type 16 [Homo sapiens]
- gi|402 epidermal cytokeratin 2 [Homo sapiens]
- gi|933 2-phosphopyruvate-hydratase alpha-enolase [Homo sapiens]

**Mascot Score Histogram**

Ions score is \(-10 \times \log(P)\), where P is the probability that the observed match is a random event.

Individual ions scores > 40 indicate identity or extensive homology (p<0.05).

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

**Peptide Summary Report**

<table>
<thead>
<tr>
<th>Significance threshold ( p &lt; )</th>
<th>0.05</th>
<th>Max. number of hits</th>
<th>AUTO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard scoring</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MudPIT scoring</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ions score or expect cut-off</td>
<td>12</td>
<td>Show sub-sets</td>
<td>0</td>
</tr>
<tr>
<td>Show pop-ups</td>
<td></td>
<td>Sort unassigned</td>
<td></td>
</tr>
<tr>
<td>Suppress pop-ups</td>
<td></td>
<td>Require bold red</td>
<td></td>
</tr>
</tbody>
</table>

**Error tolerant**

1. gi|62414289 Mass: 53676 Score: 289 Matches: 25(10) Sequences: 24(9) emPAI: 1.44 vimentin [Homo sapiens] Check to include this hit in error tolerant search

**Query Observed Mr(expt) Mr(calc) Delta Mis Scor Expec Ran Uniqui Peptide**

<p>| | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>58</td>
<td>523.7864</td>
<td>1045.558</td>
<td>1045.522</td>
<td>0.0357</td>
<td>0</td>
<td>24</td>
<td>3.7</td>
<td>1</td>
<td>U</td>
<td>K.LQEMLQ.R.E</td>
<td></td>
</tr>
<tr>
<td>64</td>
<td>538.7519</td>
<td>1075.489</td>
<td>1075.496</td>
<td>0.0076</td>
<td>0</td>
<td>39</td>
<td>0.1</td>
<td>1</td>
<td>U</td>
<td>R.DNLAEDIMR.L</td>
<td></td>
</tr>
</tbody>
</table>
7.3.3. Keratin

- gi|7331218  Mass: 66149  Score: 282  Matches: 23(8)  Sequences: 21(7)  emPAI: 0.97
- keratin 1 [Homo sapiens]

Check to include this hit in error tolerant search
### 7.3.4. Cytokeratin

<table>
<thead>
<tr>
<th>Query</th>
<th>Observed Mr (expt)</th>
<th>Mr (calc)</th>
<th>Delta Mis</th>
<th>Score</th>
<th>Expect</th>
<th>Rank</th>
<th>Unique Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>416.742 831.4709 831.4814</td>
<td>0.0105</td>
<td>0</td>
<td>29</td>
<td>1.1</td>
<td>1</td>
<td>U.K.SISISVAR.G</td>
</tr>
<tr>
<td>32</td>
<td>437.757 873.5005 873.4920 0.0086</td>
<td>0</td>
<td>45</td>
<td>0.037</td>
<td>1</td>
<td>R.SLVLGGGK.S</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>487.265 972.5163 972.5240</td>
<td>0.0077</td>
<td>0</td>
<td>48</td>
<td>0.014</td>
<td>1</td>
<td>K.IKISELNR.V</td>
</tr>
<tr>
<td>81</td>
<td>571.264 1140.513 1140.512 0.0015</td>
<td>0</td>
<td>25</td>
<td>1.8</td>
<td>1</td>
<td>R.DYQEMNMTK.L</td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>590.307 1178.600 1178.593 0.0069</td>
<td>0</td>
<td>53</td>
<td>0.0034</td>
<td>2</td>
<td>K.YEELQVAGR.H</td>
<td></td>
</tr>
<tr>
<td>106</td>
<td>633.330 1264.646 1264.629 0.0015</td>
<td>0</td>
<td>24</td>
<td>3.4</td>
<td>1</td>
<td>R.TNAENEFVTIK.K</td>
<td></td>
</tr>
<tr>
<td>114</td>
<td>650.765 1299.516 1299.522</td>
<td>0.0060</td>
<td>0</td>
<td>57</td>
<td>0.0006</td>
<td>1</td>
<td>K.NMQDMEYDR.N</td>
</tr>
<tr>
<td>117</td>
<td>651.846 1301.678 1301.707</td>
<td>0.0291</td>
<td>0</td>
<td>57</td>
<td>0.0015</td>
<td>1</td>
<td>R.SLDLSIAEVK.A</td>
</tr>
<tr>
<td>140</td>
<td>436.880 1307.621 1307.646</td>
<td>0.0260</td>
<td>2</td>
<td>17</td>
<td>14</td>
<td>3</td>
<td>R.NKYEDENEK.R</td>
</tr>
<tr>
<td>142</td>
<td>692.352 1382.689 1382.630 0.0068</td>
<td>0</td>
<td>31</td>
<td>0.57</td>
<td>1</td>
<td>K.SLNNQFASFDK.V</td>
<td></td>
</tr>
<tr>
<td>153</td>
<td>738.374 1474.733 1474.741</td>
<td>0.0081</td>
<td>0</td>
<td>36</td>
<td>0.17</td>
<td>1</td>
<td>K.WELQQVDTSTR.T</td>
</tr>
<tr>
<td>154</td>
<td>738.398 1474.782 1474.779 0.0040</td>
<td>0</td>
<td>31</td>
<td>0.63</td>
<td>1</td>
<td>R.FLEQQVQLQT.K</td>
<td></td>
</tr>
<tr>
<td>156</td>
<td>738.883 1475.751 1475.725 0.0260</td>
<td>(17)</td>
<td>14</td>
<td>1</td>
<td>U.K.WELQQVDTSTR.T + Deamidated (NQ)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>158</td>
<td>508.599 1522.775 1522.781</td>
<td>0.0056</td>
<td>1</td>
<td>18</td>
<td>11</td>
<td>1</td>
<td>R.LLRDYQEMNMTK.L</td>
</tr>
<tr>
<td>178</td>
<td>533.949 1598.826 1598.826</td>
<td>0.0003</td>
<td>1</td>
<td>27</td>
<td>1.6</td>
<td>1</td>
<td>K.NKLNDLDALQAK.E</td>
</tr>
<tr>
<td>182</td>
<td>546.949 1637.827 1637.852</td>
<td>0.0253</td>
<td>1</td>
<td>63</td>
<td>0.0003</td>
<td>1</td>
<td>K.SLNNQFASFDKVR.F</td>
</tr>
<tr>
<td>185</td>
<td>829.392 1656.771 1656.785</td>
<td>0.0144</td>
<td>0</td>
<td>30</td>
<td>0.7</td>
<td>1</td>
<td>R.SGGGFSSGSAGIINQR.R</td>
</tr>
<tr>
<td>194</td>
<td>858.933 1715.851 1715.8430 0.0079</td>
<td>0</td>
<td>38</td>
<td>0.11</td>
<td>1</td>
<td>K.QISNLQGSSIDAEQR.G</td>
<td></td>
</tr>
<tr>
<td>215</td>
<td>647.992 1940.956 1940.980</td>
<td>0.0241</td>
<td>1</td>
<td>12</td>
<td>40</td>
<td>1</td>
<td>U.K.LNDLDALQAKEDLAR.L</td>
</tr>
<tr>
<td>220</td>
<td>665.316 1992.927 1992.969</td>
<td>0.0421</td>
<td>0</td>
<td>(51)</td>
<td>0.0049</td>
<td>1</td>
<td>U.R.THNLQFESFINNL.R</td>
</tr>
<tr>
<td>222</td>
<td>665.327 1992.961 1992.969</td>
<td>0.0076</td>
<td>0</td>
<td>73</td>
<td>3.5e-05</td>
<td>1</td>
<td>U.R.THNLQFESFINNL.R</td>
</tr>
<tr>
<td>230</td>
<td>728.711 2183.113 2183.118</td>
<td>0.0004</td>
<td>2</td>
<td>25</td>
<td>21</td>
<td>1</td>
<td>U.K.NKLNDLDALQAKEDLAR.L</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Query</th>
<th>Observed Mr (expt)</th>
<th>Mr (calc)</th>
<th>Delta Mis</th>
<th>Score</th>
<th>Expec</th>
<th>Rank</th>
<th>Unique Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>449.2052896 3958896.4062</td>
<td>0.0100</td>
<td>0</td>
<td>50</td>
<td>0.004</td>
<td>8</td>
<td>U.R.MTLDDFR.I</td>
</tr>
</tbody>
</table>
### 7.3.5. Actin

<table>
<thead>
<tr>
<th>Query</th>
<th>Observed Mr(expt)</th>
<th>Mr(calc)</th>
<th>Delta</th>
<th>Miss Score</th>
<th>Expect</th>
<th>Rank</th>
<th>Unique</th>
<th>Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>78</td>
<td>566.7750</td>
<td>1131.5354</td>
<td>1131.5197</td>
<td>0.0158</td>
<td>0</td>
<td>46</td>
<td>0.02</td>
<td>U</td>
</tr>
<tr>
<td>84</td>
<td>581.3056</td>
<td>1160.5671</td>
<td>1160.6111</td>
<td>0.0144</td>
<td>0</td>
<td>43</td>
<td>0.037</td>
<td>1</td>
</tr>
<tr>
<td>93</td>
<td>599.8476</td>
<td>1197.6807</td>
<td>1197.6982</td>
<td>0.0175</td>
<td>0</td>
<td>27</td>
<td>1.3</td>
<td>1</td>
</tr>
<tr>
<td>166</td>
<td>506.2385</td>
<td>1515.6938</td>
<td>1515.6954</td>
<td>0.0016</td>
<td>0</td>
<td>56</td>
<td>0.0018</td>
<td>1</td>
</tr>
<tr>
<td>201</td>
<td>895.9445</td>
<td>1789.8744</td>
<td>1789.8846</td>
<td>0.0102</td>
<td>0</td>
<td>73</td>
<td>3.6e-05</td>
<td>1</td>
</tr>
</tbody>
</table>
7.3.6. Activation of neutrophils with fMLF

Flow cytometric analysis of neutrophils treated with fMLF. A) An fMLF titration was performed to examine at what concentration maximal neutrophil activation would be achieved as seen by increased CD11b expression. B) 1μM fMLF was incubated with neutrophils for increasing amounts of time to examine at what time point maximal shedding of CD62-L was accomplished as another marker of activation.
7.3.7. Myeloperoxidase assay

The presence of myeloperoxidase in each fraction was tested by using O-dianisidine. An absorbance at 450nm is proportional to the activity of myeloperoxidase indicating most azurophilic granules were in the initial 5 fractions.
References

References


References


References


References

References


References

139. Lorenz, N., The role of staphylococcal superantigen-like protein 7 (SSL7) in immune evasion in Doctor of Philosophy, Molecular Medicine and Pathology 2012, University of Auckland, New Zealand.


References
References


References

References

References

