

Streptococcal pathogens express surface proteins that target inhibitory LLR and complement regulating factors

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Abstract

Background: *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Streptococcus pneumonia* are major causes of invasive human diseases. Bacteria must evade host immune responses to promote virulence utilising mechanisms such as targeting host inhibitory immune receptors and complement regulators. Whilst inhibitory leukocyte Ig-like receptors (LILR) are known targets of bacteria, the bacterial ligands are unknown. Similarly, streptococcal pathogens are known to target the complement regulator factor H (FH), but the mechanism of immune evasion remains unclear. Thus, the hypothesis proposed was that streptococcal pathogens target inhibitory LILR and FH to enhance bacterial immune evasion.

Method: A combination of biochemical and cellular assays using isogenic mutants were used to identify streptococcal ligands that interact with LILR. A panel of recombinant proteins and bacterial mutants were constructed to identify bacterial ligand binding domains in LILR and FH. Finally, ex vivo whole blood and neutrophil assays were performed to assess the functional consequences of the bacterial engagement with LILR and FH.

Results: This study identified the first bacterial ligands that bind inhibitory LILR: *S. pyogenes* M3 protein binds LILRB2 and *S. agalactiae* β protein binds LILRB3. Data also suggest that *S. pneumoniae* bind LILRB3 via a subset of PspC proteins. Two LILRB3 binding domains were identified in β protein – B75KN and B6C – and a FH binding domain in a loop in B75KN. The data suggest that β protein inhibits neutrophil phagocytosis through engagement with LILRB3, but not by sequestering FH. In contrast, M3 engagement of LILRB2 does not enhance immune evasion; however, recombinant LILRB2 inhibited M3-mediated *S. pyogenes* adhesion to host cells.

Conclusion: Streptococcal pathogens express surface proteins that directly interact with inhibitory LILR. *S. agalactiae* β protein engages LILRB3 potentially promoting immune evasion and sequesters FH inhibiting complement. In contrast, host cells may secrete LILRB2 to bind M3 and inhibit *S. pyogenes* adhesion and colonisation.

Table of contents

Streptococcal pathogens express surface proteins that target inhibitory LILR and complement regulating factors	1
Declaration of originality	2
Copyright declaration	2
Acknowledgements	3
Abstract	4
List of figures	9
List of tables.....	10
Chapter 1. Introduction	11
1.1 Bacterial infections and the innate immune system	11
1.1.1 Bacterial infections.....	11
1.1.2 Immune system.....	11
1.1.3 Main components of the innate immune response.....	12
1.2 Immune receptors sense microbes and signalling	19
1.2.1 Toll like receptor (TLR) family and MyD88-mediated activating signalling.....	20
1.2.2 FcR and ITAM-mediated activating signalling	22
1.3 Immunomodulation and inhibitory receptors.....	23
1.3.1 Inhibitory receptors	23
1.3.2 ITIM-mediated signalling.....	26
1.3.3 Modulation of inhibitory receptors.....	27
1.3.4 Paired inhibitory and activating receptors modulate immune cell responses.....	27
1.4 Leukocyte Ig-like Receptors (LILR) as modulators of innate immune cell activity.....	28
1.4.1 LILR polymorphisms are associated with inflammatory diseases	29
1.4.2 Structure of LILR family of receptors	30
1.4.3 Expression of LILR family of receptors	31
1.4.4 Inhibitory LILR and their endogenous ligands.....	33
1.4.5 Inhibitory LILR regulation of neutrophil and macrophage effector functions	37
1.5 Targeting of inhibitory receptors by human bacterial pathogens.....	43
1.5.1 Direct interaction of human bacterial pathogens with inhibitory receptors	44
1.5.2 Functional impact of inhibitory receptor and bacteria interactions	46
1.6 Human bacterial pathogens target inhibitory LILR.....	47
1.6.1 Pathogen ligands of inhibitory LILRs	47
1.6.2 Functional consequences of inhibitory LILR and pathogen interactions	48
1.7 Streptococcal pathogens	49
1.7.1 <i>Streptococcus agalactiae</i>	50
1.7.2 <i>Streptococcus pyogenes</i>	53

1.7.3 <i>Streptococcus pneumoniae</i>	56
1.8 Hypothesis, aims, objectives	59
Chapter 2. <i>S. pyogenes</i> engages the inhibitory LILRB2 receptor via the M3 protein	60
2.1 Introduction.....	60
2.1.1 Hypothesis and aims	62
2.2 Materials and methods	62
2.2.1 Bacterial strains and growth conditions.....	62
2.2.3 Construction of expression vectors.....	64
2.2.4 Expression and purification of recombinant M3 protein	66
2.2.5 Biochemical assays.....	67
2.2.6 Bacterial cell culture assays	68
2.2.7 Ex vivo human blood assays.....	70
2.3 Results	71
2.3.1 LILRB2 interacts with <i>emm3 S. pyogenes</i> strains	71
2.3.2 <i>S. pyogenes</i> bind LILRB2 through a trypsin sensitive surface protein	73
2.3.3 Expression of M3 protein at the bacterial surface is necessary for LILRB2 interactions.....	76
2.3.4 Bacterial expression of M3 protein is sufficient for LILRB2 binding.....	77
2.3.5 LILRB2 directly binds M3 protein	79
2.3.6 M3-mediated engagement of LILRB2 does not enhance bacterial survival in whole blood	82
2.3.7 Soluble LILRB2 may block M3-mediated bacterial adhesion	83
2.4 Discussion	87
2.5 Supplementary figures	90
Chapter 3. <i>S. agalactiae</i> engages the inhibitory LILRB3 receptor via β protein	91
3.1 Introduction.....	91
3.1.1 Hypothesis and Aims.....	93
3.2 Materials and methods	93
3.2.1 Bacterial strains and growth conditions.....	93
3.2.2 Constructing vectors	101
3.2.3 Expression of recombinant bacterial proteins	111
3.2.4 Expression of recombinant LILR proteins.....	112
3.2.5 Construction of <i>L. lactis</i> heterologous expression system	112
3.2.6 Culture of LILR-expressing human cell lines.....	112
3.2.7 Biochemical assays.....	113
3.2.8 Bacterial cell culture assays	114
3.2.9 Human cell culture assays.....	115
3.2.10 Generating predicted β protein molecular structures	116
3.3 Results	116
3.3.1 <i>S. agalactiae</i> β protein is a LILRB3 ligand.....	117

3.3.2 Engagement of LILRB3 by β protein causes LILRB3 cross-linking	122
3.3.3 LILRB3 binds B6C and B75KN-CTD domains within the β protein	123
3.3.4 Defining the LILRB3 binding interface on the β protein	127
3.3.5 Constructing β -expressing <i>L. lactis</i> with E357 and L218 residues mutated	131
3.3.6 Blocking β protein binding to LILRB3 enhances neutrophil phagocytosis of <i>L. lactis</i>	133
3.4 Discussion	134
Chapter 4. Characterising <i>S. agalactiae</i> β protein binding to Factor H and its contribution to immune evasion	137
4.1 Introduction	137
4.1.1 Hypothesis and aims	140
4.2 Material and methods	140
4.2.1 Bacterial strains and growth conditions	140
4.2.2 Vector construction	141
4.2.3 Expression and purification of recombinant β protein	143
4.2.4 Biochemical assays	143
4.2.5 Producing a β heterologous expression model using <i>L. lactis</i>	144
4.2.6 Bacterial complement assays	144
4.2.7 Human serum and <i>ex vivo</i> human blood assays in <i>L. lactis</i>	146
4.3 Results	147
4.3.1 The B75KN domain of β contains a FH binding motif	147
4.3.2 A disordered loop in B75KN-CTD (FHBD) is essential for FH binding	148
4.3.3 Surface expression of β protein is sufficient to confer FH binding to <i>L. lactis</i>	150
4.3.4 FH bound to β protein retains co-factor activity	153
4.3.5 β expression reduces C3b deposition	155
4.3.6 Functional consequences of β -FH on immune evasion	157
4.4 Discussion	159
Chapter 5. LILRB3 binds a subset of <i>S. pneumoniae</i> PspC proteins	163
5.1 Introduction	163
5.1.1 Hypothesis and aims	165
5.2 Materials and methods	165
5.2.1 Bacterial strains and growth conditions	165
5.2.2 Construction of PspC His-tag vectors	167
5.2.3 Expression of recombinant bacterial proteins	169
5.2.4 Biochemical assays	170
5.2.5 Bacterial cell culture assays	170
5.2.6 Human cell culture assays	171
5.2.7 Bioinformatical analysis	172
5.3 Results	175
5.3.1 The CTD of B75KN shares structural and sequence homology to PspC proteins of <i>S. pneumoniae</i>	175

5.3.2 LILRB3 binds to a subset of recombinant PspC proteins	179
5.3.3 <i>S. pneumoniae</i> TIGR4 does not interact with LILRB3 to enhance adhesion.....	184
5.4 Discussion	188
Supplementary figure 5.1	191
Chapter 6. Discussion and future work.....	202
6.1 Summary of investigation.....	202
6.2 General Summary	205
6.3 Future work	206
6.3.1 How do bacteria target inhibitory LILRB2 and LILRB3?	206
6.3.2 Do <i>S. pyogenes</i> and <i>S. agalactiae</i> interact with inhibitory LILRB2 and LILRB3, respectively, for immune evasion purposes?	208
6.3.3 What are the signalling pathways triggered by bacteria binding to inhibitory LILR?.....	211
6.3.4 Do other bacteria interact with inhibitory LILR?	212
6.3.5 Do bacteria regulate LILRB3 expression?	213
6.3.6 What is the role of bacteria sequestering FH?	214
6.3 Conclusion	215
References	215

List of figures

Figure 1.1. Schematic showing representative innate immune cell interactions with bacteria.....	13
Figure 1.2. Illustration of neutrophil mechanisms of microbial killing.	15
Figure 1.3. The complement system.....	18
Figure 1.4. An immune cell expressing TLR4 binds to LPS expressed on microbial cell surface.	21
Figure 1.5. Inhibitory signalling in immune cells by though ITAM-mediated and MyD88 -mediated mechanisms.	26
Figure 1.6. The leukocyte receptor complex (LRC).	29
Figure 1.7. LILR family of receptors.	31
Figure 1.8. Inhibitory LILR host ligands.	34
Figure 1.9. LILR expression in activating and resting neutrophils.....	39
Figure 1.10. Inhibitory LILR interactions with pathogens.	48
Figure 2.1. <i>S. pyogenes</i> carrying emm3 gene bind LILRB2.....	72
Figure 2.2. LILRB2.His interacts with <i>S. pyogenes</i> M3-serotype surface protein(s), that are blocked by collagen.	75
Figure 2.3. Construction of <i>L. lactis</i> expressing M3 protein.	77
Figure 2.4. LILRB2 directly binds M3-His protein.	80
Figure 2.5. Blocking <i>S. pyogenes</i> and <i>L. lactis</i> interaction with LILRB2 does not enhance bacterial survival in whole blood.....	82
Figure 2.6. Recombinant LILRB2 inhibits M3-expressing bacterial adhesion to Collagen I and IV.	85
Supplementary Figure 2.1: Pure recombinant LILR-His and LILR-Fc.	90
Figure 3.1: <i>S. agalactiae</i> binds to rLILRB3-Fc via the β protein.	118
Figure 3.2. Surface β protein expression is sufficient for LILRB3 binding to bacterial surfaces.	121
Figure 3.3. LILRB3 engagement suppresses immune function.	123
Figure 3.4. LILRB3 binds two independent domains in the β protein.....	125
Figure 3.5. Defining the LILRB3 binding interface on the β protein.....	128
Figure 3.6. β protein residues involved in LILRB3 binding are distally located suggesting a mechanism for LILRB3 clustering.	131
Figure 3.7. Mutation of E357 and L218 residues on β protein is not sufficient to abrogate LILRB3 binding.	132
Figure 3.8. β protein interaction with LILRB3 suppresses neutrophil phagocytosis.	134
Figure 4.1. FH is a major complement regulator.	138
Figure 4.2. B75KN of the β protein contains a factor H (FH) binding motif.	148
Figure 4.3. FH binds a disordered loop in B75KN-CTD.....	149
Figure 4.4. Surface expression of β protein is sufficient to confer FH binding to <i>L. lactis</i>	152
Figure 4.5. FH bound to β protein retains co-factor function.....	154
Figure 4.6. β expression reduces C3b deposition.	156
Figure 4.7. B75KN domain attenuates neutrophil phagocytosis and whole blood survival independently of FH binding.	158
Figure 5.1 B75KN-CTD shares structurally homology to PspC, a highly diverse <i>S. pneumoniae</i> cell wall protein.	176
Figure 5.2 PspC subtypes express domains homologous to clade consensus sequences.	180
Figure 5.3 LILRB3 binds specific PspC subtypes.	182
Figure 5.4 PspC3 mediates <i>S. pneumoniae</i> adherence to THP-1 cells independent of LILRB3.	185
Figure 6.1 Thesis summary diagram.	206

List of tables

Table 1.1 Expression profile of inhibitory receptors on myeloid cells.	24
Table 1.2. Expression profile of LILR on myeloid cells.	32
Table 1.3 Bacterial ligands that interact with inhibitory immune receptors.	44
Table 2.1. Bacterial strains used in Chapter 2.....	63
Table 3.1. <i>S. agalactiae</i> strains used in Chapter 3.	93
Table 3.2. <i>L. lactis</i> strains used in Chapter 3.....	95
Table 3.3. <i>E. coli</i> strains used in Chapter 3.	96
Table 3.4. Vectors used in Chapter 3.	102
Table 3.5. Primers used in Chapter 3.	106
Table 3.6. Cell lines used in Chapter 3.	113
Table 4.1. Bacterial strains used in Chapter 4.....	141
Table 4.2. Vectors used in Chapter 4.	142
Table 5.1. Bacterial strains used in Chapter 5.....	165
Table 5.2. Vectors used in Chapter 5.....	167
Table 5.3. Primers used in Chapter 5.....	168
Table 5.4. Human cell lines used in Chapter 5.....	171
Table 5.5. Protein sequences used in Chapter 5.....	172

Chapter 1. Introduction

1.1 Bacterial infections and the innate immune system

1.1.1 Bacterial infections

Bacterial infections are a huge global health burden that disproportionally affects developing nations. Indeed, bacteria can cause a range of life-threatening diseases such as sepsis, meningitis, and pneumonia. A 2019 systematic analysis of global bacterial infections estimated that 13.7 million deaths were attributed to bacterial infections, with 4.95 million deaths associated with bacterial antimicrobial resistance (AMR) (Naghavi et al. 2022). Thus, developing the use of effective therapeutics is essential to reduce global mortality and morbidity rates, and promote global health equality. Though the development of antibiotics and vaccines therapeutic has greatly reduced mortality and morbidity rates, the rise of AMR and lack of effective vaccines for many bacterial pathogens has limited global efforts to reduce, prevent, and treat bacterial infections. The development of new antibiotics is being outpaced by the rise of AMR and for those few bacterial vaccines available, their effectiveness at eliciting protective immunity is gradually being eroded, due to increased selection pressure of non-vaccine-covered isolates. Thus, innovative strategies for developing therapeutics to reduce the burden of bacterial infections on human health are required.

One promising strategy is to discover bacterial ligands that directly interact with the human immune system to evade immune responses. Identification of these bacterial factors, and elucidation of the immune evasion mechanisms, provide an opportunity to discover novel bacterial therapeutic targets and/or host factors that can be targeted to enhance immune response to bacterial infections. Targeting bacterial ligands may sensitise the bacteria to clearance by classical antibiotics, whilst host-directed strategies can boost immune cell activity and enhance immune cell recruitment to sites of infection (Munguia and Nizet 2017). Thus, investigating host-pathogen interactions, and determining the ligands and mechanisms involved, provides an innovative new strategy for producing novel therapeutics that may reduce the global health burden of bacterial infections.

1.1.2 Immune system

Humans are under constant threat from bacterial, viral, fungal and protozoan pathogens and rely upon the immune system to protect them from infectious diseases. However, activation of the immune system comes at the cost of damaging host tissue and utilising resources (Chen et al. 2017). Therefore, a delicate balance must be maintained for optimal immune homeostasis: the immune system must be activated quickly to kill harmful microbes, whilst it must also be suppressed quickly to prevent damage to the host. The immunoregulatory mechanisms that suppress immune responses provide an opportunity for pathogens to exploit and promote their own survival.

The immune system is composed of two main arms: the innate immune system and the adaptive immune response. Innate immunity is composed of four types of defensive barriers: (1) physical barriers, such as the skin and mucosa, (2) physiological, such as temperature or pH, (3) innate immune cells such as phagocytes, (4) inflammatory molecules such cytokines and complement. Adaptive immunity is composed of specialised immune cells - T and B cells. The major differences between the innate and adaptive immune systems are their speed and method of immune recognition: the innate immune response is quick and recognises common pathogen-associated molecular patterns (PAMPs), whilst the adaptive response is slower and recognises antigens specific to individual infectious pathogens (Turvey and Broide 2010). The innate immune response is performed by immune cells and humoral factors that act within minutes to mount a robust inflammatory response to infectious pathogens. The adaptive immune response can take a week to activate and expand T and B lymphocyte cells to clear pathogens.

External facing surfaces such as the skin and the gastrointestinal (GI) tract are constantly exposed to microbes that must be prevented from access to sterile internal environments such as the circulatory system. These surfaces act not only as a physical barrier but also as a physiological barrier, being maintained in acidic environments to suppress the growth of microbes; for example, skin is maintained at pH 3-5 (Marshall et al. 2018). If anatomical barriers such as the skin and mucosal surfaces fail to prevent microbes from invading the host, the next line of defence is the innate immune cells and inflammatory molecules. Phagocytes, with the aid of inflammatory molecules, sense and kill pathogens and serve to activate the adaptive immune response (Marshall et al. 2018). However, pathogens have evolved mechanisms to suppress these immune cells and inflammatory responses thereby suppressing innate immune action, and subsequent adaptive immune responses.

1.1.3 Main components of the innate immune response

Innate immune cells are white blood cells that mediate innate immunity. They include granulocytes (neutrophils, basophils, eosinophils), macrophages, monocytes, dendritic cells, mast cells, and natural killer (NK) cells (Turvey and Broide 2010). Certain innate immune cells, such as macrophages and dendritic cells, reside in local tissues for two purposes (1) to directly kill microbes, and (2) to recruit circulating immune cells to the site of infection

(Guilliams et al. 2014). The activation of such “resident” immune cells induces signals that lead to the recruitment of several immune cells that are in circulation to sites of infection including neutrophils, basophils, eosinophils, monocytes, and NK cells (**Fig 1.1**) (Marshall et al. 2018). These immune cells perform a wide range of functions and play specific roles in detecting and eliminating different pathogen types. For example, neutrophils are primarily effective at clearing bacterial and fungal microbes, whilst NK cells are specialised for clearing virally infected cells (Mantovani et al. 2011).

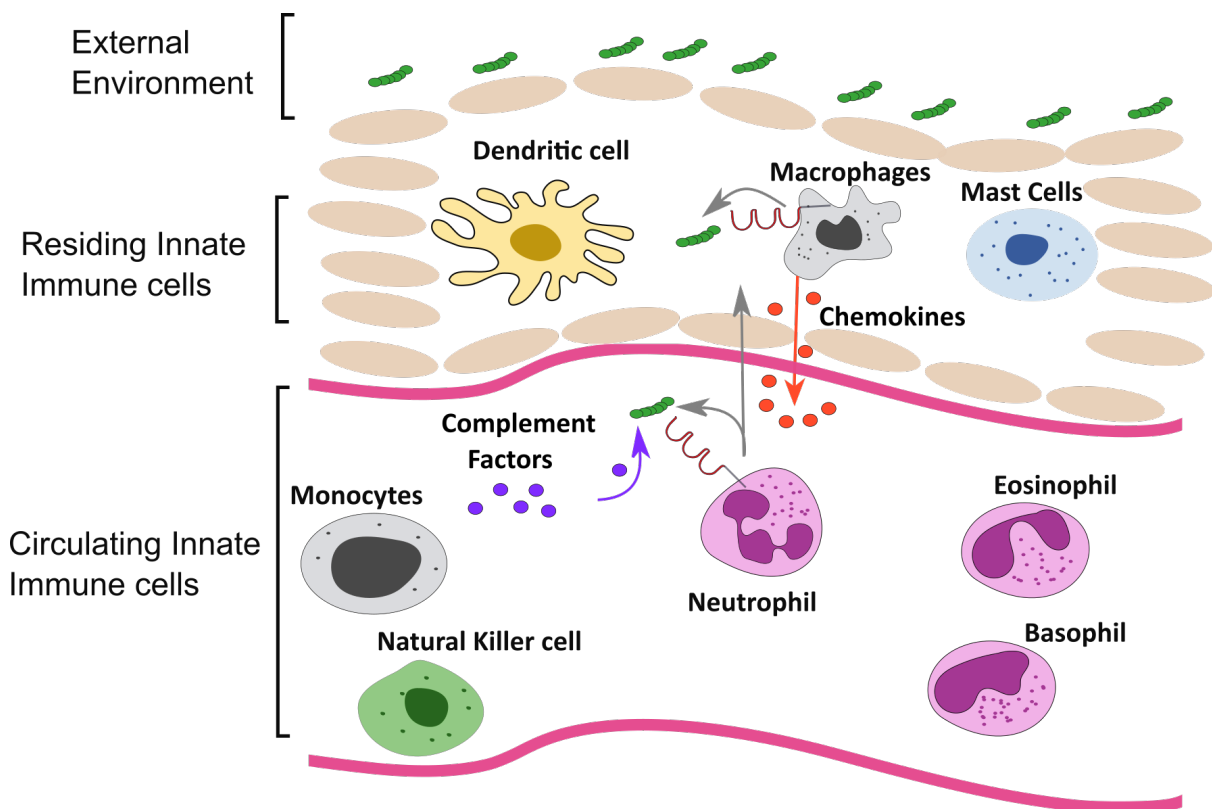


Figure 1.1. Schematic showing representative innate immune cell interactions with bacteria. Grey arrows highlighting interactions of residing innate immune cells (macrophages) and circulating immune cells (neutrophils) with bacteria. Resident immune cells such as macrophages, mast cells, and dendritic cells encounter pathogens first, recognising pathogens via receptors express on their cell surfaces and directly killing pathogens. Upon recognition of pathogens, resident immune cells also secrete chemokines into circulation (red arrow) enabling recruited of immune cells that reside in circulation to sites of infection. Circulating immune cells such as neutrophils, eosinophils, basophils, monocytes, and natural killer cells respond to chemokines and quickly translocate to sites of infection to aid clearance of pathogens. These immune cells are also important for clearance of pathogens that have invaded the circulatory system and are aided in recognising and killing pathogens by circulating complement factors (purple arrow).

1.1.3.1 Neutrophils

Neutrophils are by far the most abundant immune cells in circulation and act as first responders to bacterial infections. Once bacteria have invaded host tissue, immune cells such as macrophages and dendritic cells secrete proinflammatory cytokines and chemokines that alert circulating neutrophils that there is an invading microbe. In response to these chemoattractants, neutrophils quickly extravasate from blood vessels to the site of infection in response to certain proinflammatory factors such as tumour necrosis factor (TNF), type I and II interferons (IFNs) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Mantovani et al. 2011). These factors serve not only to recruit neutrophils but also activate them and prolong their survival at sites of infection (Mantovani et al. 2011). In addition, complement system activation (described below) produces factors such as C5a that also serve to recruit neutrophils to sites of infection and promote inflammation (Price et al. 2015). Upon entering the site of infection, neutrophils are highly effective at killing extracellular bacteria through three main effector functions: phagocytosis, degranulation, and NETosis (**Fig 1.2**). Phagocytosis of bacteria involves the engulfment of bacteria within cell membranes forming phagosomes that fuse to lysosomes, resulting in bacterial degradation by reactive oxygen species (ROS) and other microbicidal molecules such as myeloid peroxidase, lysozyme, and phospholipase (Kobayashi and DeLeo 2009). Degranulation describes the process through which neutrophils secrete antimicrobial molecules and peptides from granules stored in the cytosol. Neutrophils contain azurophil (primary), specific (secondary), and gelatinase (tertiary) granules and secretory vesicles that contain antimicrobial molecules and express regulatory receptors on their membranes (Cowland and Borregaard 2016; Lacy 2006). Upon degranulation, granules translocate to the cell membrane and phagosomes releasing antimicrobial peptides, proteins, and degradative enzymes that kill extracellular and phagocytosed microbes. NETosis is characterised by the release of DNA and protein complexes (NETs) that form mesh-like structures that trap and kill bacteria (Yipp and Kubes 2013; Apel, Zychlinsky, and Kenny 2018). NETs are formed of decondensed chromatin and granule proteins, and they are particularly important for the degradation of microbes that cannot be phagocytosed. However, NETosis comes at a high cost to the cell with rupture of the cell membrane resulting in cell death.

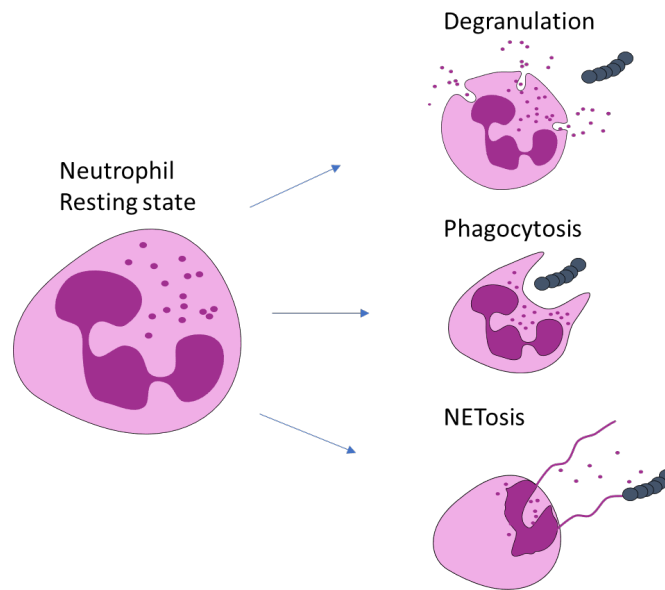


Figure 1.2. Illustration of neutrophil mechanisms of microbial killing. Phagocytosis, degranulation, and NETosis all contribute to the antibacterial effector functions of neutrophils.

Whilst these neutrophil effector functions are highly effective at clearing bacterial pathogens they also result in damage to host cells and tissue (Chan et al. 2021). Indeed, dysfunction or deregulation of neutrophil functions can result in severe autoimmune diseases. For example, the accumulation of NETs drives the development of systemic lupus erythematosus (SLE), a severe autoimmune disease. Another important example is rheumatoid arthritis (RA) which is characterised by joint damage caused by excessive inflammation. During RA there is an abundance of neutrophils in the synovial tissue and fluid, and they are thought to initiate and progress the disease through secretion of proinflammatory factors and release of NETs (Fu et al. 2021). Thus, neutrophil effector functions must be tightly regulated to prevent inappropriate damage to host tissue and cells and maintain healthy levels of neutrophils to prevent future infections.

1.1.3.2 Macrophages

Macrophages are another key phagocyte that are important for innate immune responses to bacterial pathogens. Similar to neutrophils, macrophages kill microbes through phagocytosis and secrete cytokines to recruit additional immune cells to sites of infection, but additionally present antigens to activate adaptive immune cells (Hirayama, Iida, and Nakase 2017). Macrophages reside within tissues or circulate in the blood in their precursor form as monocytes. Tissue-residing macrophages exhibit different phenotypes depending on their environment, and as such are categorised into different subsets; for example, alveolar macrophages in the lung, Kupffer cells in the liver, osteoclasts in bone, and adipose tissue macrophages in fat (Zhang, Yang, and Ericsson 2021; Yu et al. 2018). Their importance in antibacterial defence is demonstrated by numerous studies

showing that macrophage depletion enhances the susceptibility of mice to experimental infections (Hur et al. 2021; Lee et al. 2020; Goldmann et al. 2009).

Macrophages are highly plastic cells that play different roles as mediators of inflammation depending on their environment. They are generally classified into M1 (pro-inflammatory phenotype) or M2 (immunosuppressive phenotype) macrophages based on gene expression and surface markers. For example, MHC class II and CD14 are surface markers of M1 macrophages, whilst CD36 and CD206 are surface markers of M2 macrophages (Ross, Devitt, and Johnson 2021). Whether macrophages polarise to M1 or M2 type is determined by the signals they sense from the external environment. M1 macrophages differentiate from monocytes exposed to Th1 cytokines, such as TNF- α and IFN- γ , and/or bacterial ligands such as LPS. Upon M1 activation, they subsequently express a range of proinflammatory factors including IL-1 β , IL-6, IL-12 and TNF- α that serve to activate and recruit immune cells to sites of infection (Ross, Devitt, and Johnson 2021). In contrast, M2 macrophages differentiate from monocytes exposed to Th2 cytokines such as IL-4, IL-13, and IL-10 (Hirayama, Iida, and Nakase 2017; Ross, Devitt, and Johnson 2021). Upon M2 activation, they secrete anti-inflammatory cytokines IL-10 and TGF- β that suppress immune cell functions. Thus, M1 macrophages are important for propagating the inflammatory response to infectious pathogens, whilst M2 macrophages are associated with suppressing inflammation which is key for the resolution of the inflammatory response and tissue repair. Importantly, macrophages polarised to M1 or M2 phenotypes are not fixed in their phenotype and can be subsequently repolarised to another phenotype in response to the environment (Zhang, Yang, and Ericsson 2021).

Dysfunction or deregulation of macrophage activity increases susceptibility to infectious pathogens and the risk of autoimmune diseases. Mouse models with reduced alveolar macrophages show increased susceptibility to *Streptococcus pneumoniae* infection with nearly a 10-fold higher bacterial burden 3 hours post-infection (Deady et al. 2014). Abrogating macrophage functions also increases susceptibility to other important bacterial pathogens such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* (Park et al. 2022). In contrast, chronic macrophage activation can result in several autoimmune disorders including atherosclerosis, multiple sclerosis, and rheumatoid arthritis (Ross, Devitt, and Johnson 2021). Both human and mouse *in vivo* models have demonstrated that chronic activation of M1 macrophages by iron overloading resulted in unrestrained release of proinflammatory factors such as ROS and TNF- α that damage neighbouring tissue resulting in impaired wound healing (Sindrilaru et al. 2011). Thus, macrophage activity must be tightly regulated to maintain healthy immune homeostasis and prevent disease.

1.1.3.3 Complement system

Innate immune cells are supported in their antimicrobial activity by the complement system. The complement system is composed of factors in human serum that 'complement' the antimicrobial activity of immune cells. The importance of this host defence system is demonstrated by the discovery of analogues of the complement

system in distant species in the horseshoe crab (*Carcinoscorpius rotundicauda*) suggesting an ancient common ancestor utilised a complement system that has been retained in its descendants due to a strong evolutionary pressure (Zhu et al. 2005). Indeed, complement is essential for the effective clearance of pathogenic microbes in humans playing important roles in opsonising microbes, lysing microbes, and promoting inflammation. The complement system achieves these functional outcomes by activation of 3 distinct pathways: the classical pathway, lectin pathway, and alternative pathway (**Fig 1.3**). The three complement pathways are activated by distinct mechanisms and proteolytic cascade pathways that converge on C3 and C5 convertase molecules. These convertases produce the end products that promote inflammation, lyse microbes, and/or opsonise microbes (**Fig 1.3**).

The classical pathway is driven by antibody opsonisation of microbes. It is activated when C1q, in complex with C1r and C1s serine proteases, binds the Fc region of antibodies deposited on microbes (Kojouharova, Reid, and Gadjeva 2010). Autocatalysis of the serine proteases results in cleavage of C4 and C2 to produce C4a, C4b, C2a, and C2b. C4b and C2a fragments bind to form a C3 convertase (C4bC2a) on microbial surfaces, that converts C3 into C3b and C3a (Dunkelberger and Song 2010). C3b binds to the microbial surface, opsonising the microbe for recognition by immune cells. In addition, C3b binds C4bC2a to form a C5 convertase (C4bC2aC3b) that cleaves C5 to C5a and C5b and initiates the formation of the membrane attack complex (MAC) (Carlin, Chang, et al. 2009). MAC is a membrane pore-forming complex that lyses Gram-negative microbe surfaces. The C3a and C5a fragments produced are potent anaphylatoxins that promote inflammation. Thus, activation of the classical pathway initiates a series of proteolytic cascades resulting in opsonisation and lysis of microbes, and promotion of further inflammation.

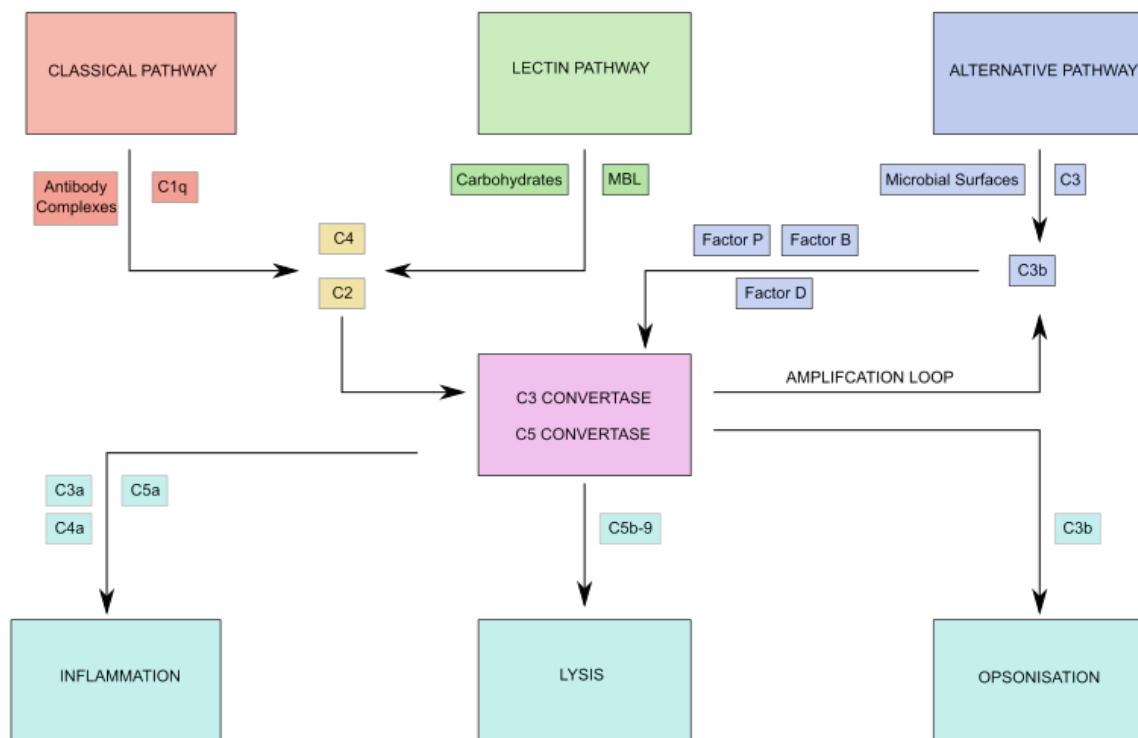


Figure 1.3. The complement system. The classical, lectin and alternate pathways are depicted with key factors involved and processes highlighted (adapted from Dunkelberger and Song 2010). The classical pathway is activated by C1q binding to the Fc region of antibodies deposited on microbes resulting in proteolytic cascades involving C4 and C2, that result in the formation of C3 and C5 convertase. The lectin pathway is activated by mannose-binding lectin (MBL) binding carbohydrates expressed on microbial surfaces, resulting in proteolytic cascades involving C4 and C2 to produce C3 and C5 convertases. The alternative pathway is activated by C3 hydrolysis to C3b and subsequent deposition onto microbial surfaces, resulting in a proteolytic cascade involving Factor P, Factor B, and Factor D, which in turn produces C3 and C5 convertase. Importantly, the alternative pathway contains an amplification loop. C3b generated positively feeds back into the alternative pathway to produce more C3b. C3 and C5 convertase produced by the classical, lectin, and alternative pathway promotes inflammation by the production of anaphylatoxins C3a and C5a, directly lyse microbes through the formation of the membrane attack complex (MAC, C5b-9) on microbial surfaces, and opsonise microbes for phagocytosis by immune cells through C3b deposition on microbial surfaces.

The lectin pathway follows a similar proteolytic cascade to the classical pathway. However, it is initiated by pattern recognition receptors (PRR) that recognise carbohydrates expressed on microbial surfaces (**Fig 1.3**). A single PRR can recognise PAMPs on several sugars that decorate microbial surfaces. For example, mannose-binding lectin (MBL) is a receptor that binds mannose, glucose, fructose, and *N*-acetyl-D-glucosamine (Turner 2003). The diversity of sugars recognised by MBL enables activation of the lectin pathway in response to bacteria, viruses, fungi, and protozoa (Turner 2003). MBL activation results in association with MBL-associated serine proteases (MASPs)-1, -2, and -3 (Harboe and Mollnes 2008) that act in a similar fashion to C1r and C1s to trigger

cleavage of C4 and C2 (Carlin, Chang, et al. 2009). Thus, the lectin pathway acts in the same manner as the classical pathway but is triggered in a C1 and antibody-independent manner.

Unlike the classical and lectin pathways, the alternative pathway is constantly active at low levels due to the spontaneous hydrolysis of C3 into C3b in a 'tick over' system. C3b deposited on microbial surfaces cleaves factor B to form the C3 convertase (C3bBb) (Harboe and Mollnes 2008). C3 convertase stabilised by factor D and factor P (properdin), cleaves all nearby C3 into C3b, that in turn produces more C3 convertases in an amplification loop. Despite what the name suggests, the alternative pathway is the key pathway responsible for the activation of the complement system due to this amplification loop that enables massive production of C3b for opsonisation and C3a anaphylxin. Indeed, studies comparing C5a production between complement pathways demonstrated that even after specific activation of the classical or lectin pathway, the majority of C5a produced (~80-90%) is due to alternative pathway activation (Harboe and Mollnes 2008). Because the alternative pathway is constitutively active and contains a powerful feedback loop, the levels of C3 convertase are tightly regulated by complement regulatory proteins such as factor H (FH), FH-related proteins (FHR), and decay accelerating factor (DAF) (Cho 2015). These factors negatively regulate the production of C3 convertase and C3b production, thereby inhibiting activation of the alternative pathway and more broadly the complement system.

The complement system plays a critical role in the clearance of bacterial infections. The importance of the complement system as a determinant of disease has been demonstrated by genetic association studies. Single nucleotide polymorphisms (SNP) in the FH gene are associated with a 2-fold higher susceptibility to meningococcal disease caused by *Neisseria meningitidis* in a UK Caucasian cohort (Haralambous et al. 2006). As the complement system is a highly effective antimicrobial response system, several bacterial pathogens have evolved immune evasion mechanisms that target components of the complement system. For example, streptococcal pathogens - *S. agalactiae*, *S. pyogenes* and *S. pneumoniae* – all express polysaccharide capsules that bind FH thereby reducing C3b deposition on their cell surface (Yu et al. 2018). Furthermore, streptococcal pathogens express cell wall-associated proteins that bind FH, including β protein (*S. agalactiae*), M protein (*S. pyogenes*), and PspC protein (*S. pneumoniae*) (Yu et al. 2018). The multiple strategies employed by bacterial pathogens to evade and/or subvert the complement system demonstrate its importance for innate immunity (Zipfel and Skerka 2009).

1.2 Immune receptors sense microbes and signalling

Immune receptors enable immune cells to sense and respond to microbial pathogens. Activating immune receptors either directly bind microbial ligands or microbes opsonised with antibody or complement molecules. The interaction of these activating receptors with microbial-derived or endogenously derived factors often triggers signalling events that activate immune cells and initiate antimicrobial effector functions.

Activating receptors that directly bind conserved microbial ligands called PAMPs are broadly classified as PRRs. Examples of PRR families include Toll-like receptors (TLRs), C-type lectins (CLEC), and the cytoplasmic receptors

of ribonucleic acids (RIG), and NOD-like receptors (NLRs) (Mantovani et al. 2011). TLRs are the best-characterised PRRs and will be described in detail below in section 1.2.1 as an exemplar of PRR interactions with bacterial ligands. However, other families such as the CLEC are also important for the recognition of bacteria and for driving anti-bacterial immunity. For example, Mincle is expressed on the surface of macrophages and recognises trehalose-6,6'-dimycolate expressed in the *M. tuberculosis* cell wall (Ishikawa et al. 2009).

Alternatively, activating receptors indirectly recognise microbes through antibodies and complement factors that coat microbes via immunoglobulin Fc receptors (FcR) and complement receptors (CR), respectively. The immunoglobulin Fc receptors (FcR) recognise the Fc region of different classes of antibodies, with an example discussed in detail below. FcR are classified based on the type of antibody they bind: FcγR (FcγRI, FcγRII, and FcγRIII) bind IgG; FcαR binds IgA; FcδR binds IgD; FcεR binds IgE; IgμR binds IgM (Ben Mkaddem, Benhamou, and Monteiro 2019; Hamdan, Lang, and Lang 2020). Thus, the expression of relatively few FcR enables immune cells to sense a huge range of pathogens by utilising the diversity of antibody recognition sites. Alternatively, complement factors opsonise microbes and are recognised by CR expressed by immune cells. For example, C3b is recognised by the CR1 receptor, which upon binding to C3b triggers neutrophil-mediated phagocytosis (Boero et al. 2023). Therefore, the expression of FcR and CR enables immune cells to recognise and kill a huge range of microbes.

1.2.1 Toll like receptor (TLR) family and MyD88-mediated activating signalling

The TLR family are the best-characterised PRR and will be used here as an example of PRR binding to microbial ligands. TLR are broadly expressed by innate immune cells such as macrophages, neutrophils, basophils, eosinophils, dendritic cells, and NK, adaptive immune cells such as B and T cells, and also non-immune cells such as fibroblasts and epithelial cells (Kawasaki and Kawai 2014; Duan et al. 2022). Composed of 10 subtypes (TLR1-TLR10), TLRs are transmembrane glycoproteins expressed on both cell membranes and intracellular organelles such as lysosomes (Kawai and Akira 2010). TLR members recognise a range of PAMP ligands including proteins, lipoproteins, lipids, and nucleic acids derived from bacterial, fungal, viral, and protozoal pathogens (Akira, Uematsu, and Takeuchi 2006). The TLR members can be categorised into two subgroups based on localisation and PAMP recognition: group I consists of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 that are generally localised on the plasma membrane and recognise microbial membrane components; with group II consisting of TLR3, TLR7, TLR8 and TLR9 that are localised in intracellular organelles such as lysosomes and recognise microbial DNA (Kawai and Akira 2010).

Bacterial microbes are typically sensed by TLR4, TLR2, and TLR5 which activate immune cells in a MyD88-mediated manner. TLR4 is the archetypal TLR that binds lipopolysaccharide (LPS) of the Gram-negative bacterial cell membrane (Lu, Yeh, and Ohashi 2008). Upon engagement of LPS, TLR4 dimerises allowing for recruitment of Toll/interleukin-1 receptor (TIR) domain adaptors (**Fig 1.4**), which activates myeloid differentiation factor 88

(MyD88) and MyD88 adaptor-like protein (Mal) to drive NF- κ B activation and cytokine production. Alternatively, LPS can induce TLR4 to signal via TRIF-related adaptor molecule (TRAM) and TIR-domain-containing adapter-inducing interferon- β (TRIF) (Fitzgerald et al. 2001; Skjesol et al. 2019). These signals induce phagocytosis and/or secretion of proinflammatory cytokines. Thus, a single PRR senses a huge range pathogens through recognition of LPS present in the wall of Gram-negative bacteria and triggers multiple immune responses independent of antibody or complement factors.

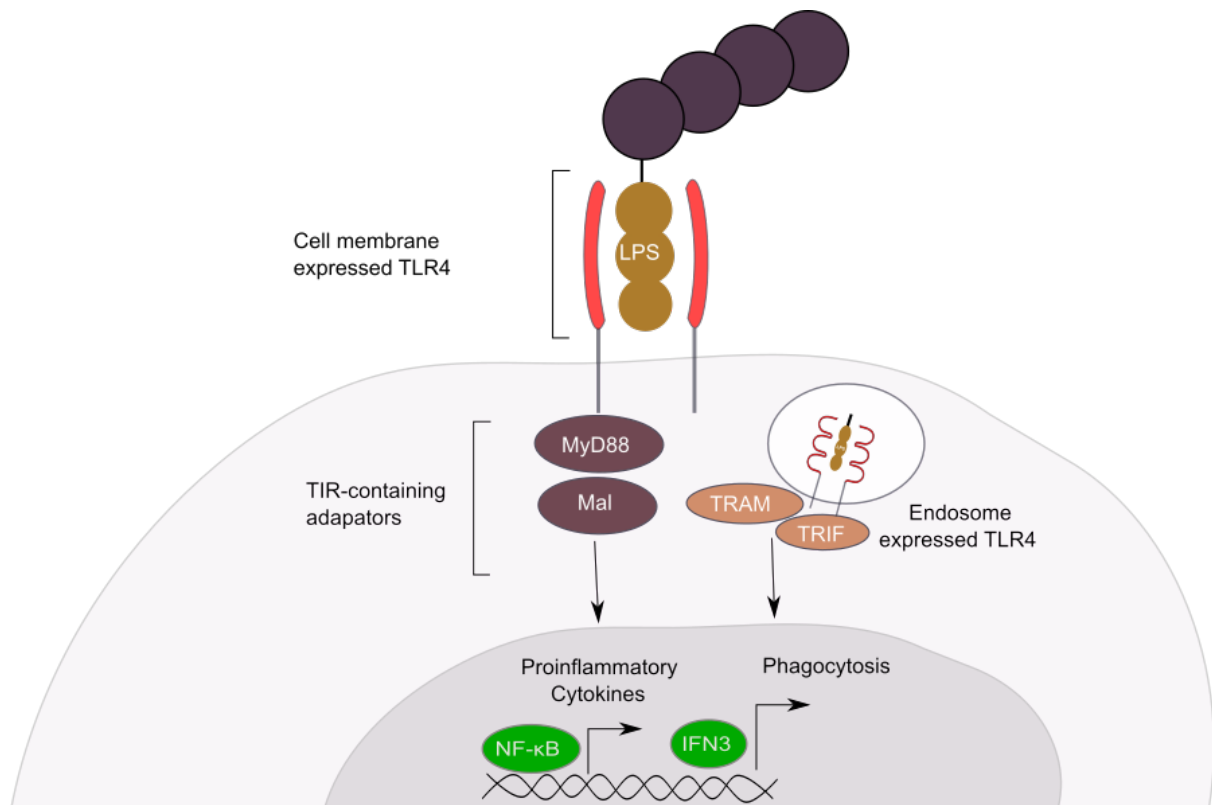


Figure 1.4. An immune cell expressing TLR4 binds to LPS expressed on microbial cell surface. The binding of LPS to TLR4 induces TLR4 dimerisation and the recruitment of TIR-containing receptors. TLR4 expressed on the cell surface induces NF κ B mediated secretion of proinflammatory cytokines. TLR4 expressed in endosomes recruits TRAM and TRIF that favour activation of IFN3 and promote phagocytosis.

TLR2 is another well-characterised TLR that complements the TLR4 receptor by recognising Gram-positive bacteria. TLR2 recognises peptidoglycan, lipoteichoic acid, and lipoproteins present in the bacterial cell wall of Gram-positive bacteria (Kawai and Akira 2010). TLR2 binding to lipoproteins present in *S. pneumoniae* was shown to promote NF- κ B mediated proinflammatory cytokine release in macrophages (Tomlinson et al. 2014).

Thus, TLR2 and TLR4 play complementary roles, with TLR2 recognising Gram-positive bacterial ligands, whilst TLR4 recognises Gram-negative bacterial ligands (Takeuchi et al. 1999).

1.2.2 FcR and ITAM-mediated activating signalling

FcRs are key immune receptors that are ubiquitously expressed by neutrophils, basophils, eosinophils, NK cells, and macrophages (Ben Mkaddem, Benhamou, and Monteiro 2019). Neutrophils are initial responders that are essential for bacterial clearance and require FcR to effectively sense a huge range of microbial pathogens. These receptors bind with high-affinity to IgG- and IgA-antigen complexes, which triggers signalling cascades and immune cell activation and effector functions. In contrast, the low affinity binding of ligands to FcRs triggers inhibitory signalling, likely to prevent the autoreactivity of immune cells to host antibodies (Ben Mkaddem et al. 2014). Of note, an inhibitory member of the FcR family – FcRIIB – exists that regulates activating signalling (Ben Mkaddem, Benhamou, and Monteiro 2019).

Most activating receptors, including FcR activating receptors, share an immunoreceptor tyrosine-based activation motif (ITAM) based on a consensus sequence of YxxL/Ix₆₋₁₂YxxL/I, where x represents any amino acid residue (Futosi, Fodor, and Mócsai 2013). ITAM-containing FcR include FcγRII (CD16), FcγRII (CD32), FcγRI (CD64) and FcαRI (CD89). FcR signalling involves ITAM-mediated phosphorylation cascades that result in immune cell activation and trigger effector functions.

Some FcRs possess a long cytoplasmic tail and their own ITAMs, whilst other FcRs co-associate with the ITAM-bearing Fc receptor common γ chain (FcRγ). Nonetheless, the signalling via endogenous or associated ITAMs results in a similar signalling cascade. As an example, FcγRIIA (CD32) expressed on neutrophil surfaces recognises IgG-antigen complexes resulting in the phosphorylation of two ITAM residues within its cytoplasmic tail (Daniels et al. 2010). Phosphorylated ITAMs activate spleen tyrosine kinase (Syk) through binding of Syk via phosphorylation of two Src-homology 2 domains (SH2) (Mócsai, Ruland, and Tybulewicz 2010). In turn, this can induce the activation of effector pathways such as phosphatidylinositol-3 kinase (PI3K), mitogen-activated protein kinase (MAPK), and nuclear factor (NF)-κB (Humphrey, Lanier, and Nakamura 2005). Once activated these ITAM-induced pathways result in the translocation of proinflammatory transcription factors such as NF-κB to the nucleus that control immune cell effector functions such as phagocytosis and secretion of proinflammatory cytokines such as IL-1 and TNF-α (Lawrence 2009; Zhu, Yue, and Wang 2014).

In addition to certain FcR, several activating receptors from other immune receptor families induce intracellular signalling by associating with FcRγ. This includes Mincle (CLEC4E), Dectin-2, MAIR-IV and IL-3R (Hamerman et al. 2009). Mincle is a C-type lectin receptor expressed by innate immune cells including neutrophils and macrophages that recognises both fungal and mycobacterial structures (Futosi, Fodor, and Mócsai 2013). Upon recognition of PAMPs, Mincle associates with FcRγ propagating ITAM signalling thereby increasing the production of proinflammatory cytokines (Yamasaki et al. 2008). Thus, FcR receptors are incredibly important

for immune cells to recognise potential pathogens, not only recognising a huge range of microbes through antibody-opsonisation but also acting as an adaptor protein for activating receptors from additional families.

1.3 Immunomodulation and inhibitory receptors

The innate immune system is essential for responding quickly to pathogenic bacteria and prevent infectious diseases. However, inappropriate immune activation can result in damage to host tissue and can lead to autoimmune diseases (Cahilog et al. 2020; Goodnow 2007). This is particularly a problem if immune cells are activated at the wrong time or in the wrong place. Notably, hyperactivation of immune cells can result in the secretion of proinflammatory factors and proteases that damage the surrounding tissue causing autoimmune disorders. For example, systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disorder that results in damage to many organs including skin, joints, the central nervous system, and kidneys (Kaul et al. 2016). SLE patients that fail to clear NETs by DNase I exhibit high levels of autoantibodies against NETs, and these immune-complexes drive inflammation mediated by dendritic cells, resulting in host tissue damage and SLE disease progression (Garcia-Romo et al. 2011). In addition, proinflammatory factors damaging host tissue, hyperactivation of certain effector functions such as NETosis results in neutrophil cell death; and therefore, over-activation or continuous activation of neutrophils eventually depletes total neutrophil numbers and may leave the host susceptible to future infections (Cahilog et al. 2020). Thus, immune cell activation must be finely regulated in order to elicit immunity without causing inflammatory damage.

Inhibitory receptors regulate innate immune cell activation thereby controlling inflammatory responses and reducing potential cytotoxicity. They are required to focus the immune response and prevent autoreactivity that can result in autoimmune diseases (Wang, Wang, and Gershwin 2015). Both mouse and human studies have demonstrated the importance of inhibitory receptors to prevent development of autoimmune disorders. For example, FcγRIIB knockout mice developed autoantibodies and autoimmune glomerulonephritis characteristic of SLE and arthritis (Bolland and Ravetch 2000). In addition, human association studies suggest that variant and copy number of inhibitory immune receptors are risk factors for certain autoimmune disorders such as Crohn's disease, inflammatory bowel disease, and ulcerative colitis (Olde Nordkamp, Koeleman, and Meyaard 2014). Thus, expression and function of inhibitory receptors are essential for maintaining immune homeostasis.

1.3.1 Inhibitory receptors

There are over 300 genes encoding potential inhibitory receptors in the human genome (Rumpret et al. 2020). The importance of inhibitory receptors is demonstrated by the fact that they are expressed in all immune cells. Some well-known protein families consisting of inhibitory receptors include leukocyte Ig-like receptors (LILR), CD33-related sialic acid binding immunoglobulin lectins (Siglec), leukocyte associated Ig receptor (LAIR), carcinoembryonic-antigen-related cell-adhesion molecules (CEACAM), FcR, killer Ig-like receptors (KIR), and

CD300 (Ravetch and Lanier 2000). The inhibitory receptors from these families are expressed in a range of myeloid cells such as neutrophils, eosinophils, basophils, monocytes, macrophages, mast cells, and dendritic cells which all play crucial roles in the innate immune response (**Table 1.1**). Interestingly, individual inhibitory receptors within protein families have diverse expression profiles suggesting different roles during immune homeostasis.

1 Table 1.1 Expression profile of inhibitory receptors on myeloid cells.

Inhibitory Receptors	Myeloid Expression Profile	REFERENCES
LILRB1	Macrophages, dendritic cells, and monocytes	(Abdallah et al. 2021)
LILRB2	Macrophages, dendritic cells, monocytes, neutrophils, basophils, and mast cells	(Abdallah et al. 2021)
LILRB3	Macrophages, dendritic cells, monocytes, neutrophils, basophils, eosinophils, and mast cells	(Abdallah et al. 2021)
LILRB4	Macrophages, dendritic cells, and monocytes	(Abdallah et al. 2021)
LILRB5	Monocytes	(Abdallah et al. 2021)
Siglec-1	Macrophages and dendritic cells	(Duan and Paulson 2020)
Siglec-2	Macrophages and dendritic cells	Duan and Paulson 2020)
Siglec-3	Monocytes, macrophages, neutrophils, dendritic cells	Duan and Paulson 2020)
Siglec-5	Monocytes, neutrophils, and mast cells	Duan and Paulson 2020)
Siglec-7	Monocytes and mast cells	Duan and Paulson 2020)
Siglec-8	Eosinophils, basophils and mast cells	Duan and Paulson 2020)
Siglec-9	Monocytes, neutrophils, dendritic cells	Duan and Paulson 2020)
Siglec-10	Monocytes and eosinophils	Duan and Paulson 2020)
Siglec-11	Macrophages	Duan and Paulson 2020)
Siglec-14	Neutrophils and monocytes	Duan and Paulson 2020)
Siglec-15	Macrophages	Duan and Paulson 2020)
Siglec-16	Macrophages	Duan and Paulson 2020)
CEACAM1	Neutrophils, eosinophils, basophils, dendritic cells, monocytes, and macrophages	(Horst et al. 2018)
FcγRIIB	Neutrophils, eosinophils, basophils, monocytes, macrophages, mast cells, dendritic cells.	(Bournazos, Gupta, and Ravetch 2020)
LAIR-1	Neutrophils, monocytes, eosinophils, basophils, and mast cells	(Carvalho et al. 2020; Verbrugge et al. 2006; Jin et al. 2018)
CD300a	Neutrophils, eosinophils, basophils, monocytes, and dendritic cells	(Borrego 2013)

Table 1.1 Expression profile of inhibitory receptors on myeloid cells. The myeloid cells that express inhibitory receptors from LILR, Siglec, CEACAM, FcγR, LAIR, and CD300 families.

Inhibitory receptors generally function to maintain a threshold of activation for immune cells and/or provide negative feedback for immune cell activation. The role an inhibitory receptor plays in maintaining activation thresholds and/or providing negative feedback function is suggested by their expression patterns (Rumpret et al. 2020). Constitutively expressed inhibitory receptors are likely responsible for maintaining the activation threshold (Meyaard et al. 1997). Of these ‘threshold’ receptors some can be downregulated upon immune cell activation to modulate the activation threshold. For example, the inhibitory receptor dendritic cell immunoreceptor (DCIR) is downregulated on dendritic cells in response to LPS (Bates et al. 1999). Downregulation of DCIR reduces the inhibitory signals within the cell and enhance the immunoactivity of the cells. Alternatively, inhibitory receptors that are upregulated during immune cell activation can provide negative feedback for immune cell activation. These ‘negative feedback’ inhibitory receptors include LILRB2 which is upregulated upon neutrophil activation (Lewis Marffy and McCarthy 2020). Importantly, certain inhibitory receptors can function to maintain the activation threshold and provide negative feedback. For example, LILRB3 is constitutively expressed on the surface of neutrophils, and surface expression is further upregulated upon activation (Zhao et al. 2020). Thus, inhibitory receptors help to fine-tune the activation and magnitude of immune cell responses.

One of the best-characterised inhibitory receptors is leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1; CD305). It is an inhibitory receptor expressed on the surface of a range of immune cells including neutrophils, NK cells, monocytes, T and B cells (van Rees et al. 2016; Yu et al. 2018). LAIR-1 recognises several host ligands including collagen (Lebbink et al. 2006), surfactant protein A (SpA)(Olde Nordkamp et al. 2014), epithelial cell adhesion molecule (EpCAM) (Olde Nordkamp et al. 2014), and complement component 1q (C1q) (Son et al. 2012). Through these interactions LAIR-1 can suppress immune cell activation. For example, LAIR-1 suppresses TLR4-mediated cellular activation (**Fig 1.5**) (Kumawat et al. 2019; Carvalho et al. 2020). Furthermore, LAIR-1 engagement negatively regulates neutrophil recruitment (Kumawat et al. 2019), degranulation, ROS production (Besteman et al. 2020), and NETosis (Geerdink et al. 2018). Thus, multiple immune cells utilise LAIR-1 inhibitory signalling to finely tune responses to bacteria and prevent damage to the host.

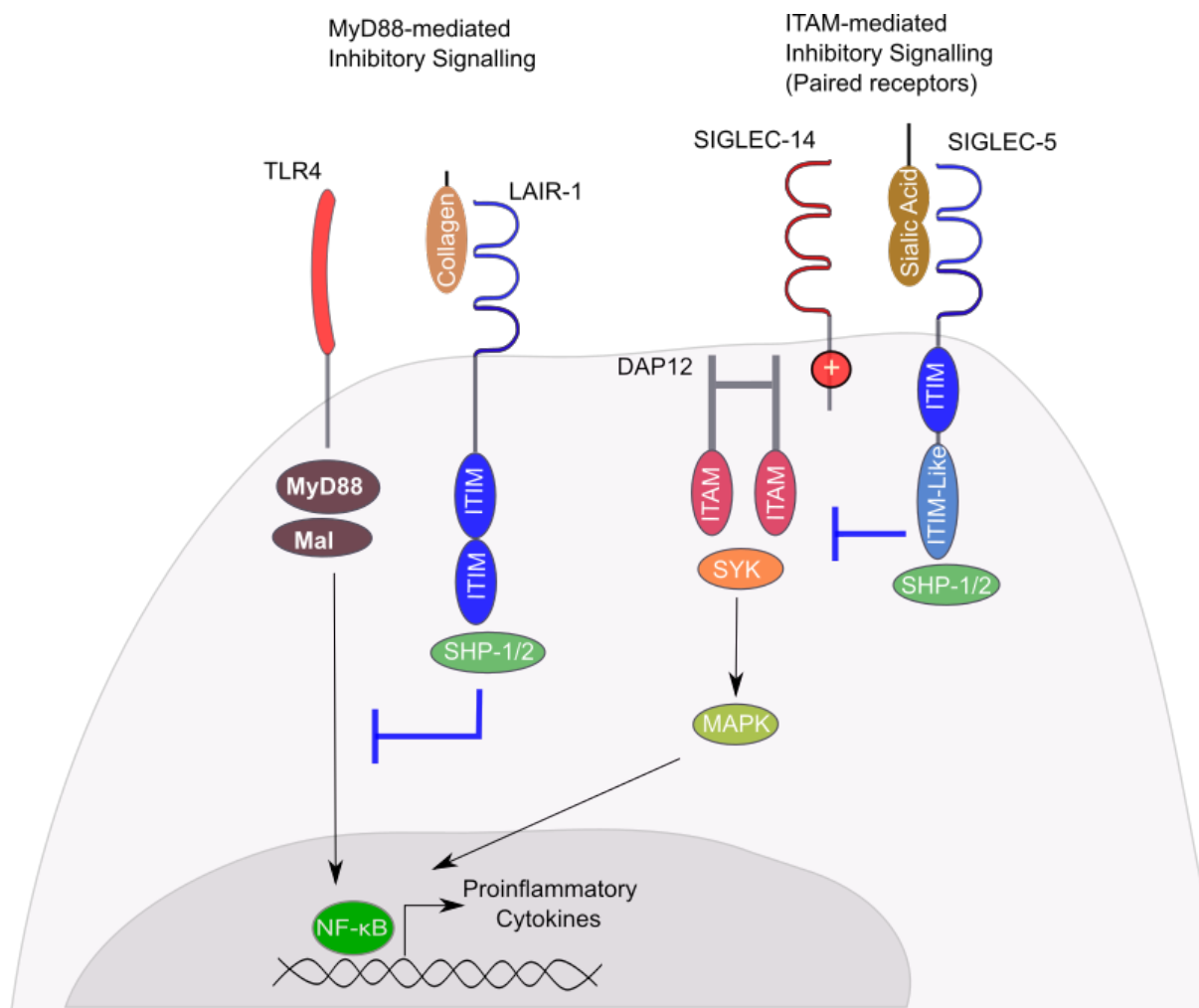


Figure 1.5. Inhibitory signalling in immune cells through ITAM-mediated and MyD88-mediated mechanisms. The inhibitory receptors Siglec-5 and LAIR-1 suppress the secretion of NF-κB mediated proinflammatory cytokine secretion. Siglec-5 inhibits the activating receptor Siglec-14, which associates with ITAM-containing DAP12, an adaptor protein that promotes activating signalling via MAPK. Siglec-5 inhibits DAP12 ITAM phosphorylation through recruiting phosphatases SHP1 and SHP2 that dephosphorylate ITAM motifs. LAIR-1 suppresses MyD88-mediated TLR4 signalling; however, the exact process is not well understood.

1.3.2 ITIM-mediated signalling

Inhibitory receptors, like LAIR-1, signal through an immunoreceptor tyrosine-based inhibitory motif (ITIM) that counter-acts the signalling of ITAM-bearing activating receptors. This provides a mechanism to regulate immune

cell activities and inflammatory responses. The ITIM motif is composed of a consensus sequence of (I/V/L/S)xYxx(I/V/L), where x represents any amino acid residue (Favier 2016).

Inhibitory receptors can suppress activating signalling through multiple distinct mechanisms. Firstly, co-clustering or co-aggregation of activating receptors with inhibitory receptors activates members of the Src family of tyrosine kinases that phosphorylate tyrosine residues within the ITIM domains (Billadeau and Leibson 2002). Phosphorylated ITIM domains subsequently recruit phosphatases SHP-1/2 or SHIP that dephosphorylate ITAM domains in nearby activating receptors. As dephosphorylated ITAM domains are inactive, intracellular signalling associated with immune cell activation and effector functions is suppressed (Getahun and Cambier 2015). Secondly, inhibitory receptors can inhibit immune signalling independently of ITAMs by targeting signalling molecules further downstream of signalling cascades. For example, several inhibitory receptors are known to suppress TLR2 and TLR4 signalling, even though these PRRs do not signal via ITAMs (Steevels and Meyaard 2011). Finally, inhibitory receptors can competitively inhibit activating receptors by binding proinflammatory factors, thereby suppressing immune cell activation (Butte et al. 2007).

1.3.3 Modulation of inhibitory receptors

Inhibitory receptor signalling and immune cell responsiveness from the environment is regulated at multiple levels. Firstly, the location and expression of inhibitory receptors can be modulated; for example, inhibitory receptors can be translocated from internal cytosolic stores to the cell membrane (Lewis Marffy and McCarthy 2020). Secondly, inhibitory receptor expression can be modulated at the transcriptional level to control overall expression; for example, LAIR-1 is differentially expressed in monocytes depending on the type of stimulation (Carvalho et al. 2020). Thirdly, the availability of inhibitory ligands can be modulated. During the initial stages of infection, the native ligands of many inhibitory receptors are often downregulated resulting in enhanced activation of immune cells and improved clearance of pathogens (Avondt, Sorge, and Meyaard 2015). Each of these mechanisms/features contributes to maintaining healthy immune homeostasis.

1.3.4 Paired inhibitory and activating receptors modulate immune cell responses

Paired receptors offer an additional mechanism to regulate immune cell activation. Inhibitory and activating receptors that recognise the same ligands are known as paired receptors (Levi-Schaffer and Mandelboim 2018). To be functionally paired both activating and inhibitory receptors must be expressed by the same cell. Having two receptors competitively binding to the same ligands, whilst promoting counter-acting signalling appears counter-intuitive. However, paired receptors are proposed to enable immune cells to recognise pathogens that target inhibitory receptors (Akkaya and Barclay 2013).

Activating paired receptors are thought to have evolved from inhibitory receptors due to their shorter evolutionary half-lives and greater gene copy number variation (Barclay and Hatherley 2008). A proposed theory for the evolution of paired receptors is that they have evolved in response to the targeting of inhibitory receptors by pathogens (Akkaya and Barclay 2013). Pathogens that have evolved to target inhibitory receptors can potentially evade immune responses by suppressing immune cell activation. Thus, in order to counterbalance this immune evasion mechanism, humans have evolved activating receptors that target pathogens. Indeed, paired receptors often exhibit high non-synonymous to synonymous SNP ratios in the human population, which is suggestive of a strong evolutionary pressure likely caused by pathogens (López-Álvarez et al. 2014; Satoh et al. 2008). However, the expression of paired receptors must be delicately balanced to promote resistance to pathogens whilst not inducing autoimmune disorders. As an example, the CD33-related sialic acid binding immunoglobulin lectins (Siglec) family contains the paired receptors Siglec-14 and Siglec-5, which modulate immune cell functions in response to the same ligands (**Fig 1.5**) (Ali et al. 2014).

The Siglec receptors are a well-characterised family of immunomodulatory receptors that recognise glycan chains containing sialic acids expressed on cell surfaces in the immune system. Humans express 13 Siglecs (Siglec-1 to Siglec-11, Siglec-14 and Siglec-15) that are expressed by most immune cells including neutrophils, macrophages, monocytes, and NK cells (Crocker, Paulson, and Varki 2007). These receptors all contain a V-set immunoglobulin domain at the N-terminal that enables binding to the sialic acid *N*-acetylneuraminic acid α 2-3 and α 2-4 linkages to galactose (Crocker, Paulson, and Varki 2007). Most Siglec function as inhibitory receptors expressing an ITIM motif and/or ITIM-like motif in their cytoplasmic tail.

Siglec-14 is considered an activating receptor that does not contain an ITIM domain and is paired with the inhibitory receptor Siglec-5. Siglec-14 has a short cytoplasmic tail and expresses an extracellular domain that shares significant homology to the Siglec-5 receptor, such that Siglec-5 and Siglec-14 are considered paired receptors (**Fig 1.5**). Whilst Siglec-14 does not contain an ITAM domain, it does express a positively charged arginine residue in the transmembrane domain that associates with the adaptor protein DAP12 (Angata et al. 2006), thus promoting activating signalling via spleen tyrosine kinase (SYK) and mitogen-activated protein kinases (MAPK) (**Fig 1.5**). Thus, Siglec-14 counterbalances the inhibitory signalling of Siglec-5 offering another layer of immune cell regulation.

1.4 Leukocyte Ig-like Receptors (LILR) as modulators of innate immune cell activity

Immunomodulatory receptors are essential for regulating innate immune cell receptors, with several families being characterised and their important functions being delineated. LILR (also referred to as Ig-like transcripts (ILT), leukocyte immunoglobulin-like receptor (LIR) and CD85) are a relatively recently discovered family of immunomodulatory that also appear to play important roles regulating innate immune cell responses (Lewis

Marffy and McCarthy 2020; Abdallah et al. 2021). LILR contain inhibitory and activating receptors that are widely expressed by myeloid lineage cell lines. The *LILR* genes are located on the leukocyte receptor complex (LRC), located on 19q13.4 human chromosome together with other important immunomodulatory families including CEACAM, Siglec, LAIR, and KIR suggesting LILRs play similar important functions (**Fig 1.6**) (Storm et al. 2021). However, whilst many of these immunomodulatory families such as those of CEACAM and Siglec families are conserved in mammals, LILRs have expanded more recently with expression being restricted to primates. Thus, LILRs represent a useful family of receptors to study more human-specific innate immune responses.

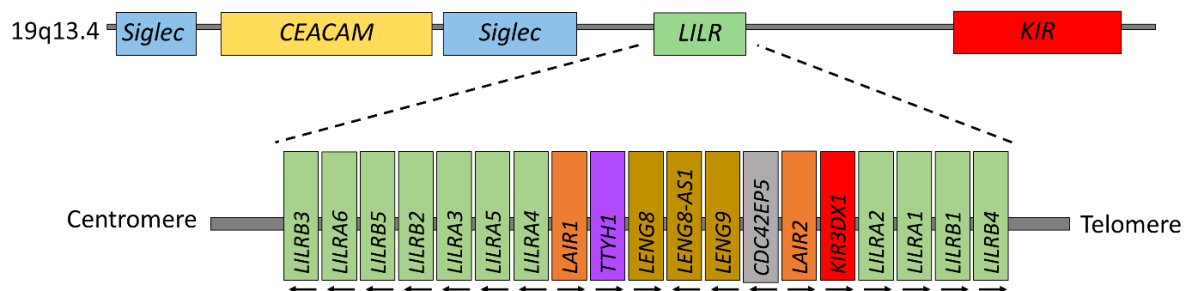


Figure 1.6. The leukocyte receptor complex (LRC). Found on human chromosome at 19q13.4, the LRC contains important immune receptor families including *SIGLEC*, *CEACAM*, *LILR*, *LAIR*, and *KIR* genes. Arrows denote the direction of transcriptional activation i.e. right (sense/positive strand) and left (antisense/negative strand).

1.4.1 LILR polymorphisms are associated with inflammatory diseases

LILR genes are rapidly evolving with single nucleotide polymorphisms (SNP) and haplotypes associated with inflammatory diseases (Hirayasu and Arase 2015). Because of the role LILR play in modulating immune activity, it is perhaps unsurprising that polymorphisms that affect LILR function or expression are associated with increased risk to inflammatory disorders.

Genetic screens have identified polymorphisms in both inhibitory and activating LILR genes that are associated with inflammatory diseases such as Takayasu arteritis (TAK), SLE, and RA. TAK is an inflammatory disease that is characterised by inflammation of the aorta, proximal branches, and pulmonary arteries which can lead to life-threatening ischemia (Dua et al. 2021). A genome-wide association study of two Takayasu arteritis cohorts from Turkey and North America identified a SNP (rs11666543) which resulted in reduced expression of LILRB3 and was associated with increased risk of disease (Renauer et al. 2015). Furthermore, a SNP (rs410852) located in the LILRA3 gene was also associated with increased risk of disease (Renauer et al. 2015).

Polymorphisms in LILRB4 and LILRA2 are associated with risk of SLE. Two functional SNP in LILRB4 (rs11540761 and rs1048801) in European-derived and Hispanic-American SLE patients were associated with increased inflammatory cytokine levels suggesting a key biological role of LILRB4 in SLE disease progression (Jensen et al. 2013). In addition, a LILRA2 SNP (rs2241524) that results in G>A mutation, is associated with SLE and microscopic polyangiitis (MPA) in a Japanese case-controlled study (Mamegano et al. 2008).

Finally, the risk of RA is associated with polymorphisms in LILRA3 and LILRB1 genes. A LILRA3 deletion polymorphism, particularly prevalent in Northeast Asian populations, results in a 6.7 kb deletion mutation that abrogates LILRA3 expression and is associated with reduced risk of RA (Du et al. 2014). In addition, a LILRB1 haplotype (LILRB1.PE01) is associated with increased risk of in a subset of RA patients (Kuroki et al. 2005). Thus, the association of LILR polymorphisms with several inflammatory diseases reflects the important roles LILR play in regulating and maintaining appropriate immune homeostasis.

1.4.2 Structure of LILR family of receptors

LILR are a family of 5 inhibitory immune receptors, 5 activating immune receptors, and 1 soluble immune receptor (**Fig 1.7**) (Lewis Marffy and McCarthy 2020; Abdallah et al. 2021). Of note, the inhibitory receptor LILRB3 and activating receptor LILRA6 are considered paired receptors. Broadly speaking, the inhibitory LILRs recognise endogenous ligands and trigger inhibitory signals that suppress the activity of innate immune cells. Therefore, inhibitory LILRs are critical regulators of innate immune responses and maintain immune homeostasis.

The 5 inhibitory LILRs are LILRB1 (also known as ILT2 or CD85j), LILRB2 (ILT4 or CD85d), LILRB3 (ILT5 or CD85a), LILRB4 (ILT3 or CD85k) and LILRB5 (CD85c) (**Fig 1.7**). The inhibitory LILRs contain 2 or 4 extracellular Ig domains, a transmembrane domain, and a long cytoplasmic tail containing ITIM residues. Upon binding of ligands to inhibitory LILRs, tyrosine residues within their ITIM domains become phosphorylated and this induces inhibitory signalling cascades (Barrow and Trowsdale 2008). The 5 activating LILRs are LILRA1 (CD85i), LILRA2 (ILT1 or CD85h), LILRA4 (ILT7 or CD85g), LILRA5 (ILT11 or CD85f) and LILRA6 (ILT8 or CD85b). The activating LILR (LILRA) contain 2 or 4 extracellular Ig domains, a transmembrane domain with a positive arginine residue, and a short cytoplasmic tail. Upon their ligation, the positively charged transmembrane domain recruits the ITAM-containing receptor FcγR. FcγR ITAM domains become phosphorylated, recruiting tyrosine kinase Syk, resulting in the stimulation of activating signalling cascades (Brown, Trowsdale, and Allen 2004; Nakajima et al. 1999). LILRA3 is a soluble immune receptor that does not contain intracellular signalling ability; however, may act to inhibit the LILRB1 and LILRB2 receptors as it recognises some of the same ligands (Lewis Marffy and McCarthy 2020).

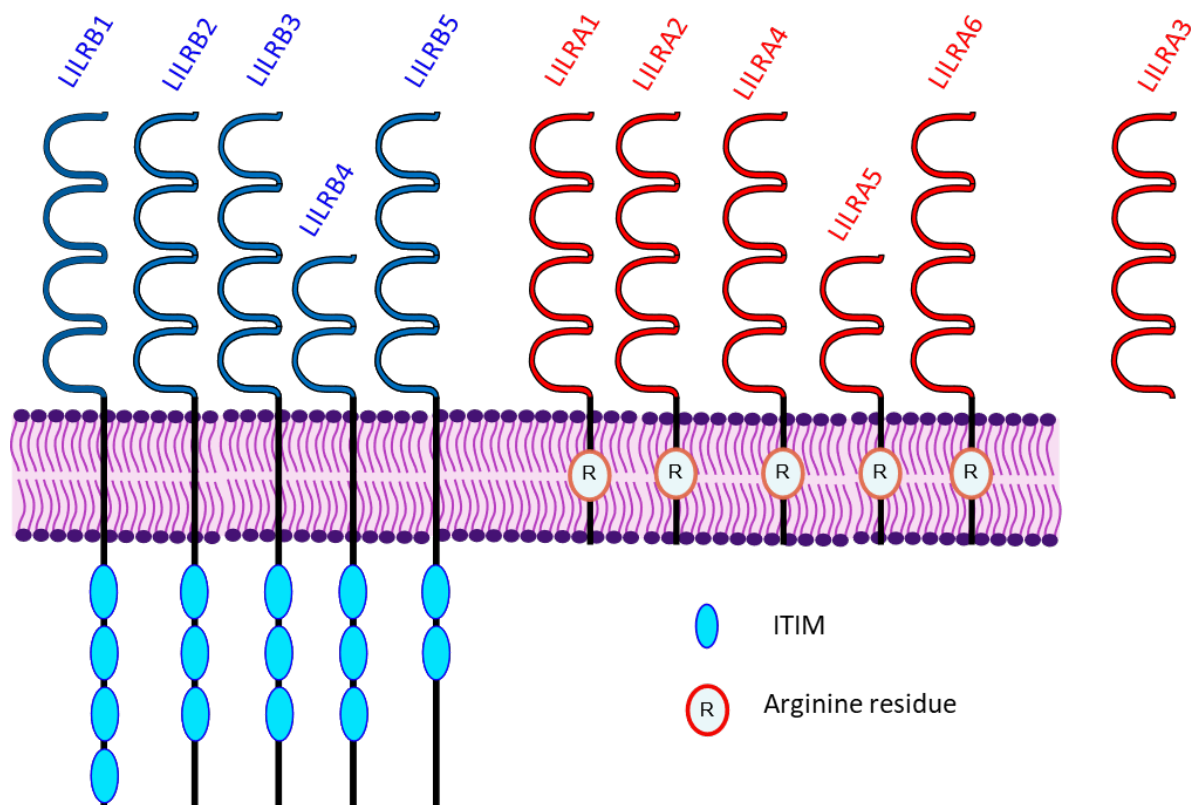


Figure 1.7. LILR family of receptors. A schematic of the LILR receptors highlighting important extra- and intra-cellular domains for regulating immune cell activation. All LILR members contain extracellular Ig-like domains that bind both common and diverse ligands. Inhibitory LILRs in blue, contain cytoplasmic tails with 2-4 ITIM domains that are crucial for intracellular signalling that suppresses immune cell activation. Activating LILRs in red, contain arginine residues in the transmembrane domain that enables association with ITAM-containing receptors, thereby promoting immune cell activation.

1.4.3 Expression of LILR family of receptors

Given that the LILR family contains several inhibitory and activating receptors, they are proposed to play a crucial role in the maintenance of immune homeostasis. They are principally thought to regulate the innate immune response due to their expression largely being restricted to innate immune cells (**Table 1.2**). Indeed, LILRs are broadly expressed by all myeloid cells including neutrophils, basophils, eosinophils, monocytes, natural killer (NK) cells, dendritic cells, mast cells and macrophages. Interestingly, the expression profile of LILRs on myeloid cells is complex as different cell types express different LILRs (**Table 1.2**). For example, whilst LILRB2 is expressed by most myeloid cells, including macrophages, dendritic cells, monocytes, neutrophils, basophils, and mast cells LILRB5 expression is restricted to monocytes and dendritic cells (Borges et al. 1997; Hogan, Jones, and Allen

2016; Samaridis and Colonna 1997; Sloane et al. 2004). In addition, some LILRs are expressed by lymphoid cells including NK cells, T cells and B cells.

Inhibitory Receptors	Myeloid Expression Profile	REFERENCES
LILRB1 (ILT2, CD85j, LIR1)	Macrophages, dendritic cells, monocytes, and neutrophils	(Samaridis and Colonna 1997; L. Borges et al. 1997; Ju et al. 2004)
LILRB2 (ILT4, CD85d, and LIR2)	Macrophages, dendritic cells, monocytes, neutrophils, basophils, and mast cells	(Samaridis and Colonna 1997; L. Borges et al. 1997; Sloane et al. 2004)
LILRB3 (ILT5, CD85a, and LIR3)	Macrophages, dendritic cells, monocytes, neutrophils, basophils, eosinophils, and mast cells	(Colonna et al. 1997; Sloane et al. 2004)
LILRB4 (ILT3, CD85k, and LIR5)	Macrophages, dendritic cells, and monocytes	(Cella et al. 1997; Ju et al. 2004)
LILRB5 (LIR-8, CD85C)	Monocytes and dendritic cells	(L. Borges et al. 1997; Hogan, Jones, and Allen 2016)
LILRA1 (CD86i, LIR6)	Monocytes and macrophages	(L. Borges et al. 1997)
LILRA2 (ILT1, CD85h, and LIR7)	Neutrophils, basophils, eosinophils, monocytes, macrophages, and dendritic cells	(L. Borges et al. 1997; Sloane et al. 2004)
LILRA3 (ILT6, CD85e, and LIR4)	Neutrophils and monocytes	(L. Borges et al. 1997)
LILRA4 (ILT7, CD85g)	Dendritic cells	(Ju et al. 2004)
LILRA5 (ILT11, CD85f, and LIR9)	Neutrophils and monocytes	(Luis Borges, Kubin, and Kuhlman 2003; Grabowski et al. 2019)
LILRA6 (ILT8)	Monocytes and neutrophils	(Grabowski et al. 2019; Bashirova, Apps, et al. 2014)

Table 1.2. Expression profile of LILR on myeloid cells.

1.4.4 Inhibitory LILR and their endogenous ligands

Due to the ubiquitous expression of the inhibitory LILRs in myeloid cells and their proposed regulatory function, identification of the endogenous ligands has become a priority for understanding how innate immune homeostasis is maintained. A broad spectrum of host ligands has been identified for inhibitory LILRs (**Fig 1.8**). Most of the endogenous ligands identified interact with LILRB1 or LILRB2, and the functional consequences of these ligand-receptor interactions on signalling and/or immune cell functions are not well understood (Abdallah et al. 2021). For those that have been studied, binding of endogenous ligands to an inhibitory LILR often suppresses immune cell responses (Zhao et al. 2020; Favier 2016; Fanger et al. 1998). This is proposed to function to prevent autoreactivity and maintain immune homeostasis.

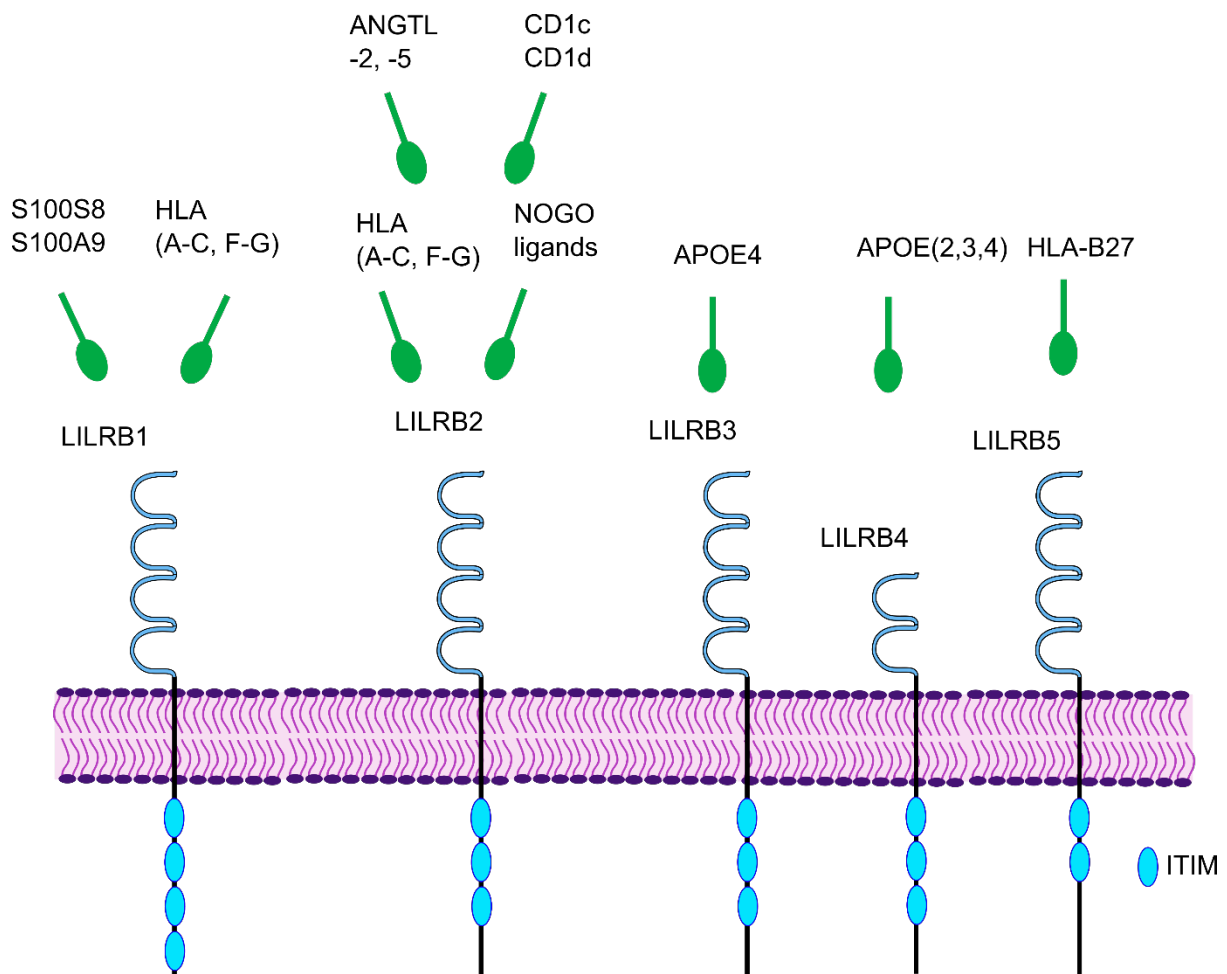


Figure 1.8. Inhibitory LILR host ligands. LILRB1 binds damage associated proteins S100S8/9 and HLA molecules (HLA A-C, F-G). LILRB2 binds HLA molecules (HLA A-C, F-G); MHC-like molecules, CD1c and CD1d; endothelial growth factors, ANGTL-2 and ANGTL-5; NOGO66, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp). LILRB3, LILRB4, and LILRB5 each bind one host ligand: LILRB3 and LILRB4 bind ApoE; whilst LILRB5 binds HLA-B57.

LILRB1 and LILRB2 recognise non-classical HLA molecules

LILRB1 and LILRB2 interactions with HLA molecules are the best characterised inhibitory LILR interactions. LILRB1 and LILRB2 broadly bind classical and non-classical HLA molecules, in both *cis* and *trans* interactions (Barkal et al. 2018; Baudhuin et al. 2013; Abdallah et al. 2021). These LILRs are grouped into the Group I LILR based on the presence of highly conserved LILRB1 residues that form the HLA-binding domain (Yu et al. 2018). The crystal structure of LILRB1 in complex with HLA-A2 (Yu et al. 2018) and LILRB2 in complex with HLA-G have been solved (Yu et al. 2018). They demonstrated that LILRB1 and LILRB2 exhibit overlapping, but distinct modes, of HLA recognition. For example, domain 1 (D1) and D2 of LILRB1 recognise the $\alpha 3$ domain and $\beta_2 m$ domain of HLA-A2 whilst the D1 and D2 domains of LILRB2 recognise the $\alpha 3$ domain of HLA-G (Shiroishi et al. 2003). The strength

of HLA binding to LILRB1 and LILRB2 depends on HLA subtype and allele, LILR polymorphisms, and the antigen being presented (Yu et al. 2018; Shiroishi et al. 2003).

The interactions of LILRB1 and LILRB2 with HLA suppress immune responses through several mechanisms. Firstly, the engagement of HLA molecules triggers inhibitory signalling in LILRB1- and LILRB2-expressing immune cells such as neutrophils and monocytes (Favier et al. 2010; Fanger et al. 1998). Secondly, the interaction of LILRB1 and LILRB2 with HLA molecules in *trans* competitively inhibits proinflammatory factors engaging with HLA molecules and subsequent T cell activation (Anderson and Allen 2009). Thirdly, LILRB2 binding to HLA molecules in *cis* suppresses immune cell functions by competitively inhibiting HLA interactions with proinflammatory ligands (Masuda et al. 2007). Finally, LILRB2 engagement with HLA can suppress antigen presentation in antigen-presenting cells, and subsequently suppress their ability to activate adaptive immune cells such as T cells (Bashirova, Martin-Gayo, et al. 2014).

Given that LILRB1 and LILRB2 interact with HLA and modulate antigen-presentation, these interactions have important consequences for disease outcomes. The best characterisation has been presented in the context of human immunodeficiency virus type 1 (HIV-1) infections. HIV infects CD4⁺ T cells of the immune system resulting in chronic disease. A study of 5,126 HIV-1 patients found that the strength of LILRB2 binding to HLA-B molecules associated with increased viral replication of HIV-1 (Bashirova, Martin-Gayo, et al. 2014) and with disease progression (Jinghe Huang et al. 2009). Because LILRB2 interactions with HLA-B inhibit antigen-presentation of dendritic cells and T-cell proliferation, polymorphism in HLA-B impacts the interaction with LILRB2 and innate immune activity against HIV-1. Thus, LILRB1 and LILRB2 interactions with non-classical HLA are crucially important for immune homeostasis and have important roles in regulating immune responses in disease situations.

Additional endogenous ligands of LILRB1

In addition to binding non-classical HLA molecules, LILRB1 binds the damage-associated proteins S100A8 and S100A9 (Arnold et al. 2013). S100A9/A8 are calcium-binding proteins constitutively expressed by monocytes and neutrophils that are secreted during inflammation. S100A8/9 release functions to promote leukocyte recruitment and cytokine release (Wang et al. 2018). Thus, increases in availability of S100A8/9 can lead to increases in LILRB1 ligation on immune cells, and subsequent suppression of the cellular activity of the immune cell.

Additional endogenous ligands of LILRB2

Additional LILRB2 host ligands include MHC class I-like proteins CD1c and CD1d (Li et al. 2009); ligands involved in neural function β amyloid, NOGO66, myelin-associated glycoprotein (MAG), oligodendrocyte myelin

glycoprotein (OMgp), and semaphorin-4A (Takeda and Nakamura 2017; Kim et al. 2013; Lu et al. 2018); complement split products C3d, C4b, C3b, iC3b and C4d (Hofer et al. 2016); endothelial growth factors angiopoietin 2 (ANGTL-2) and 5 (ANGTL-5). The functional consequences for immune cells of these interactions are not well characterised compared with interactions with HLA molecules. The exception is LILRB2 binding to CD1c and CD1d MHC class I-like proteins. CD1c and CD1d are isoforms of MHC class I-like proteins expressed by monocytes that present lipid antigens to a subset of T cells. Through their investigations, the Xu group demonstrated that LILRB2 expressed by PBMCs directly bound CD1d and CD1c inhibiting lipid antigen presentation and suppressing T cell responses (Li et al. 2009; 2012). Thus, LILRB2, similar to LILRB1, is a versatile receptor that binds a range of ligands to modulate immune responses.

LILRB3 endogenous ligands

Recent research identified apolipoprotein E4 (APOE4) as a ligand for LILRB3 (Zhou et al. 2023). Apolipoproteins (APOE) act as cholesterol carrier molecules supporting lipid transport and repairing damaged tissue in the brain (Liu et al. 2013). There are 4 APOE isotypes, APOE1 to APOE4, that play distinct biological roles being involved in lipid metabolism, immune regulation, and neuronal function (Grainger, Reckless, and McKilligin 2004; K. Ali et al. 2005). A combination of biochemical assays and cryogenic electron microscopy (cryo-EM) demonstrated that the N-terminal domain of APOE4 binds directly to D2 and D4 of LILRB3 (Zhou et al. 2023). Interestingly, the interaction of APOE4 with LILRB3 expressed on the surface of microglial cells (immune cells that provide the first line of defence in the brain) induced upregulation of genes involved in interferon and cytokine signalling (Zhou et al. 2023; Dello Russo et al. 2018). These results could demonstrate the unexpected result that LILRB3 interaction with APOE4 stimulates proinflammatory immune responses. However, the expression of LILRB3's paired receptor LILRA6 was not controlled for, thus whether these effects are due to LILRA6 or LILRB3 engagement is unclear.

LILRB4 endogenous ligands

APOEs are also the endogenous ligand for LILRB4. However, in contrast to LILRB3, LILRB4 can bind multiple APOE isoforms including APOE2, APOE3, and APOE4 (Deng et al. 2018). The interaction of APOEs with LILRB4 has been shown to inhibit monocyte migration with the consequence of suppressing T cell activity and proliferation (Deng et al. 2018; Gui et al. 2019). In addition, fibronectin is a LILRB4 ligand that upon engagement with LILRB4 suppresses the secretion of proinflammatory cytokines by dendritic cells (Paavola et al. 2021). Ligands that bind LILRB3 and LILRB4 expressed on neutrophils and macrophages are yet to be determined. Whilst inhibitory signalling is likely involved, an immunosuppressive phenotype may depend on the ligand and environmental context.

LILRB5 endogenous ligands

LILRB5 specifically binds the HLA-class I molecule HLA-B27 (Zhang et al. 2015). Interestingly, LILRB5 did not bind to other HLA molecules such as HLA-A3 and HLA-B7 (Zhang et al. 2015). Whilst the functional consequences of this interaction are yet to be elucidated, LILRB5 is expressed on the surface of circulating monocytes suggesting involvement in monocyte activation and/or differentiation into macrophages.

1.4.5 Inhibitory LILR regulation of neutrophil and macrophage effector functions

Neutrophils and macrophages are key innate immune cells that are essential for effectively clearing pathogens and preventing infectious diseases. Multiple studies have demonstrated that inhibitory LILRs are expressed by neutrophils and macrophages, and serve to regulate immune cell activation and effector functions (Mori et al. 2008; Tedla et al. 2003; Baudhuin et al. 2013). To understand the role inhibitory LILRs play during infection, it is necessary to understand inhibitory LILR expression in both resting and activating immune cell states. Because both neutrophils and macrophages are highly plastic cells that can undergo significant phenotypic changes, the surface expression of inhibitory LILRs likely changes in response to activating signals (Galli, Borregaard, and Wynn 2011).

In the case of neutrophils, their activation results in a reprogramming of immune receptors expressed at the cell surface in order to elicit new responsiveness and effector functions in response to infection. This regulation is mainly achieved through the mobilisation of receptors to the cell surface and the release of receptors from the cell surface (Borregaard, Sørensen, and Theilgaard-Mönch 2007). This is because neutrophils are largely transcriptionally inactive once they enter the bloodstream. Immune receptors can be upregulated at the surface of activated neutrophils if they are expressed and stored in secretory vesicles or granules that are released upon stimulation. For example, complement receptors 1 (CR1) and 3 are initially upregulated to the neutrophil surface during degranulation (Lundahl et al. 1995). In addition, immune receptors can be downregulated at the surface of activated neutrophils if proteases released from granules cleave the ectodomains of the receptors. For example, CR1 is subsequently downregulated after the translocation of azurophil granules (Lundahl et al. 1995). Because of this dynamic process, understanding of the inhibitory receptor expression at the cell surface upon activation can provide insights into when it likely functions to regulate neutrophil activity.

Similarly, macrophages undergo significant changes during activation and polarisation. Macrophages can become polarised into proinflammatory (M1) or immunosuppressive (M2) phenotypes depending on the stimuli and environmental context. Polarisation into M1 or M2 results in a global change in transcriptional profile that includes widespread changes in immunomodulatory receptor genes. Indeed, a comparison of M1 and M2

transcriptional profiles of monocytes differentiated with IFN- γ or IL-4 showed that M1 macrophages exhibited significantly higher expression of LILRA1, LILRA2, LILR3 and LILRA5 transcripts (Beyer et al. 2012). In addition, the LILRB3 transcript was also upregulated in M1 macrophages (Beyer et al. 2012). Thus, understanding of the LILR expression under proinflammatory conditions is key to understanding their role in regulating macrophage functions during infection.

Expression of LILR on neutrophils

The expression profiles of LILR on neutrophils according to current literature and mass spectrometry analyses are shown in (**Fig 1.9**). In summary, peptides from 3 inhibitory receptors (LILRB1, LILRB2, and LILRB3) and 3 activating receptors (LILRA2, LILRA3, and LILRA5) were detected by mass spectrometry analysis of neutrophil lysates (Lewis Marffy and McCarthy 2020). LILRA6 was also detected in neutrophils in some proteome studies. However, a recent study using immunoprecipitation and mass spectrometry only detected LILRB3-specific peptides but not LILRA6-specific peptides in neutrophil lysates (Zhao et al. 2020). LILRB4, LILRA1, and LILRA4 peptides were only detected in 1 out of 11 mass spectrometry datasets available in the public domain, and LILRB5 peptides were not detected in any datasets (Lewis Marffy and McCarthy 2020). Thus, proteomic analysis indicates that LILRB1, LILRB2, LILRB3, LILRA2, LILRA4, and LILRA5 are expressed by human neutrophils.

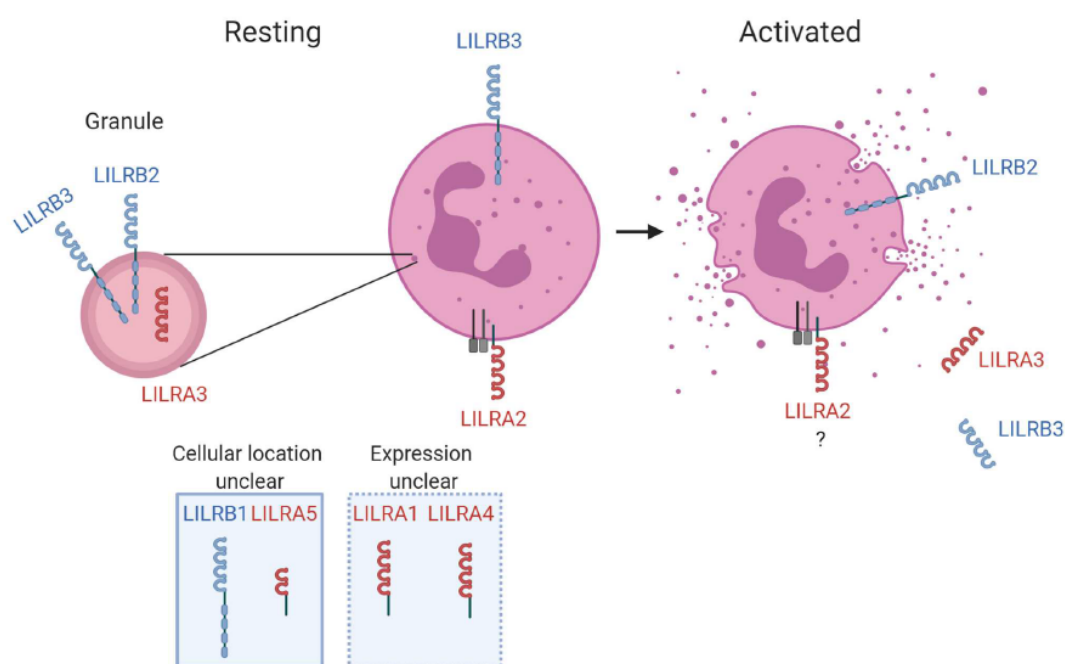


Figure 1.9. LILR expression in activating and resting neutrophils (Lewis Marffy and McCarthy 2020). A schematic showing the expression of LILR on neutrophil surfaces and/or within granules. Where expression or location of LILR is not well defined this is shown. The image was created using Biorender.

Determining the surface localisation of LILR in resting and activated neutrophils is important for understanding LILR function. Low levels of LILRB2 surface expression were reported on resting neutrophils from healthy donors using 42D1 and m402 mAb in multiple studies (Tedla et al. 2003; Yu et al. 2018; 2018; Baudhuin et al. 2013). This indicates that LILRB2 is likely expressed in granules. LILRB1 surface expression on resting neutrophils from healthy donors was detected using m401 and 293205 mAb; however, conflicting results were obtained via detection with VMP355, HP-F1, and GHI/75 mAb (Tedla et al. 2003; Pulford et al. 1991; Bankey et al. 2010; Baudhuin et al. 2013). It is possible that the latter mAbs may recognise LILRB1 epitopes that are hidden or absent when expressed on neutrophils. Thus, the exact cellular location of LILRB1 remains to be determined. LILRB3 expression on the surface of resting neutrophils has been detected by m431 and 222821 mAb (Bankey et al. 2010; Tedla et al. 2003). However, these mAb have cross-reactivity with the paired receptor LILRA6. Nonetheless, the confirmation that LILRA6 is not expressed by human neutrophils indicates that LILRB3 is expressed at the surface of resting neutrophils (Zhao et al. 2020). LILRA2 expression on the surface of resting neutrophils has been confirmed using m371 and 135 mAb clones (Hirayasu et al. 2016; Tedla et al. 2003). LILRA3 is secreted and therefore, the expression on neutrophil surfaces would not be expected. There are currently no studies showing neutrophil surface expression of LILRA5, although soluble and membrane-bound LILRA5 transcripts have been detected in neutrophils (Borges, Kubin, and Kuhlman 2003). In summary, LILRB3 and

LILRA2, and potentially LILRB1, are expressed on the surface of resting neutrophils, indicative that these LILRs likely regulate neutrophil activation by maintaining an activation threshold.

Upon neutrophil activation, LILRB3 surface expression is decreased whilst LILRB2 is upregulated (**Fig 1.9**). LILRB3 is present on resting but not activated neutrophils suggesting that LILRB3 is important for maintaining an activation threshold thereby reducing inflammatory responses that may damage host cells. In contrast, because LILRB2 is expressed on the surface of activated but not resting neutrophils, this suggests it functions to provide negative feedback and prevent neutrophil over-activation. Indeed, sepsis patients have impaired upregulation of LILRB2 upon neutrophil activation which likely contributes to the characteristic neutrophil dysfunction and deregulation (Baudhuin et al. 2013). Through these two regulatory mechanisms, LILRB2 and LILRB3 maintain neutrophil and immune homeostasis.

Functions of inhibitory LILR on neutrophils

LILRB2 and LILRB3 suppress neutrophil activation and effector functions. Utilising neutrophil and neutrophil-like cell lines the Favier group showed that LILRB2 suppressed neutrophil phagocytosis and inhibited FcγRIIa-mediated production of ROS (Baudhuin et al. 2013). Initially, they showed that LILRB2 surface expression was upregulated upon neutrophil stimulation with TNF-α, LPS, or fMLP through degranulation. Furthermore, they showed that engagement of LILRB2 using the LILRB2 ligand, HLA-G, suppressed phagocytosis and ROS production (Baudhuin et al. 2013). Interestingly, they went on to demonstrate that LILRB2 expression is inhibited during sepsis, suggesting a potential role for LILRB2 in resolving normal immune homeostasis post-infection (Baudhuin et al. 2013). Similar to LILRB2, LILRB3 suppresses neutrophil effector functions; however, LILRB3 expression levels are maintained at high levels in resting neutrophils (Lewis Marffy and McCarthy 2020). The McCarthy group utilised a mAb to cross-link LILRB3 which resulted in suppressed Fc-mediated neutrophil activation and inhibited effector functions such as phagocytosis and NETosis (Zhao et al. 2020). Furthermore, they demonstrated that mAb engagement of LILRB3 suppressed neutrophil-mediated killing of *Staphylococcus capitis*. These data suggest that LILRB2 and LILRB3 may act synergistically after neutrophil activation to regulate neutrophil effector functions.

Expression of LILR on macrophage

Macrophages express a plethora of LILR that perform regulatory roles including the inhibitory receptors LILRB1, LILRB2, LILRB3 and LILRB4; and the activating receptors LILRA1 and LILRA2 (Abdallah et al. 2021). A comprehensive study by the Takai group using mAb staining of macrophages from healthy donors showed surface expression of LILRB1 (GHI/75), LILRB2 (42D1), LILRB3 (2228321), and LILRB4 (ZM23.8) by flow cytometry using the antibodies indicated in parentheses (Mori et al. 2008). Furthermore, they showed that ITIM domains

were constitutively phosphorylated and associated with SHP-1 for LILRB1, LILRB2, LILRB3, and LILRB4 on resting macrophages (Mori et al. 2008). Thus, these inhibitory LILR receptors likely act to maintain an activation threshold to prevent autoreactivity and cytotoxicity to host cells.

In addition to being constitutively expressed, inhibitory LILRs are differentially regulated during macrophage activation. LILRB1 is downregulated on activated peripheral blood monocytes (PBMCs) in response to tetanus toxoid stimulation suggesting a role in disinhibiting the threshold of macrophage activation (Lombardelli et al. 2013). Interestingly, LILRB1 expression is dependent on the type of macrophage: decidual macrophages, important during pregnancy, exhibit lower levels of LILRB1 expression compared with PBMCs (Lombardelli et al. 2013; C. Li et al. 2009). In support of this, a study comparing LILRB1 expression in different macrophages demonstrated that LILRB1 expression was significantly different between PBMCs, splenic macrophages, ascites macrophages, and tumour-associated macrophages (TAM) (Barkal et al. 2018). LILRB1 was most highly expressed on ascites macrophages, and least on splenic macrophages (Barkal et al. 2018). This may reflect levels of macrophage activation or different functional roles depending on the environment.

LILRB2 is also differentially regulated in response to macrophage activation. LILRB2 was upregulated on macrophages in response to tetanus toxoid-stimulated macrophages, although to lower levels compared with LILRB1 (Barkal et al. 2018). Similarly, LILRB2 expression differed depending on macrophage type with almost no expression on splenic macrophages, and slightly higher levels on PBMCs, ascites macrophages, and TAMs (Barkal et al. 2018).

The expression of several LILRs is differentially modulated in PBMCs during infection and sepsis. In a purified *in vitro* system it was demonstrated that LILRB2 and LILRB4 transcripts are upregulated in macrophages in response to either LPS stimulation or *Salmonella typhimurium* infection (Brown et al. 2009). Similarly, during sepsis PBMCs upregulate expression of inhibitory LILR (Ming et al. 2019). Sepsis is a hugely complex immunological response to infection with both proinflammatory and immunosuppressive responses occurring simultaneously (Hotchkiss et al. 2009). However, the prevailing theory is the net inflammatory responses in sepsis follow a two-step process that acts like a seesaw: the initial infection causes an excessive inflammatory response, followed by an immunosuppressive stage that leaves the patient susceptible to subsequent infection (Hotchkiss et al. 2009). A study comparing transcription of *LILR* genes in PBMCs of healthy and septic patients demonstrated that LILRB3 and LILRA5 mRNA were upregulated in septic patients, 8-fold and 6-fold, respectively (Ming et al. 2019). In addition, LILRA2 mRNA was also upregulated 2-fold (Ming et al. 2019). In contrast, LILRB1, LILRB4 and LILRA4 mRNA expression was downregulated in septic patients (Ming et al. 2019). Therefore, the data suggest that inhibitory LILR may play different functional roles on macrophages during sepsis. It could mean that LILRB1 and LILRB4 are downregulated to promote macrophage activation, whilst LILRB3 may be upregulated on macrophage surfaces to prevent over-activation.

LILR expression is also determined by M1 or M2 macrophage phenotype. A study compared M1 and M2 transcriptional profiles of monocytes differentiated with IFN- γ or IL-4, respectively (Beyer et al. 2012). Using RNA-seq and qPCR they showed that M1 macrophages exhibited significantly higher expression of LILRA1,

LILRA2, LILRA3 and LILRA5 transcripts (Beyer et al. 2012). In addition, LILRB3 transcripts were upregulated in M1 macrophages (Beyer et al. 2012). Thus, data suggest that there is a general shift to increased activating LILR expression in M1 macrophages which may contribute to the proinflammatory phenotype.

Functions of inhibitory LILR on macrophage

Inhibitory LILRs play a complex role in modulating macrophage responses, with multiple studies demonstrating that the context, whether that be the microenvironment or the macrophage type, are important factors in determining the inhibitory LILR function. For example, antibody-mediated blocking of LILRB1 enhanced the capacity of macrophages to phagocytose tumour cells, suggesting LILRB1 functions in an immunosuppressive capacity (Barkal et al. 2018). Conversely, HLA-G engagement of LILRB1 on decidual macrophages upregulated secretion of proinflammatory cytokines such as IL-6 and TNF α , and this effect was blocked by anti-LILRB1 antibodies (Li et al. 2009). Thus, LILRB1 function may depend on the external environment and the type of macrophage.

Similarly, LILRB2 function appears to be context-dependent. Antibody-mediated antagonism has been shown to drive macrophage activation and inflammatory responses in the presence of macrophage colony-stimulating factor (M-CSF) (Chen et al. 2018). Furthermore, transcriptomic analysis suggests LILRB2 drives M2 maturation of macrophages (immunosuppressive phenotype) by suppressing ERK and p38 activation, and ultimately suppressing NF- κ B activation (Chen et al. 2018). However, work by the Maute group suggested that antibody blocking of LILRB2 had no inhibitory function on macrophage phagocytosis of tumour cells (Barkal et al. 2018). Thus, LILRB2 suppression of macrophage activity appears to be dependent on the stimuli and/or macrophage type.

LILRB3 functions as a clear inhibitory receptor on macrophages whose functions have important implications for both innate and adaptive immune responses. LILRB3 ligation induces inhibitory signalling and reprograms macrophages driving an M2 phenotype (Yeboah et al. 2020). As previously discussed, the comprehensive study of LILR regulation in sepsis patients by the Huang group demonstrated that LILRB3 is upregulated on PBMCs (Ming et al. 2019). This group also went on to characterise LILRB3 function in macrophages, by demonstrating that transient knock out of LILRB3 enhanced macrophage production of ROS and phagocytosis of *Pseudomonas aeruginosa* (Ming et al. 2019). Furthermore, LILRB3 suppressed the antigen presentation functions of monocytes towards T cells (Ming et al. 2019). Thus, LILRB3 plays an important role both in controlling macrophage effector functions and antigen presentation.

Finally, LILRB4 functions to suppress macrophage phagocytosis. LILRB4 regulates macrophage phagocytosis by suppressing activating FcRs. Cross-linking of LILRB4 with surface-expressed activating receptors such as Fc γ III and CR3 (CD11b) inhibits tyrosine phosphorylation and calcium mobilisation (Cella et al. 1997). Calcium

mobilisation is a biomarker for actin cytoskeleton rearrangement that is necessary for phagocytosis (Melendez 2013). Thus, LILRB4 likely suppresses macrophage phagocytosis by inhibiting FcγRIII receptor.

1.5 Targeting of inhibitory receptors by human bacterial pathogens

Human bacterial pathogens have evolved numerous immune evasion mechanisms to enhance their survival within their host. The bacterial immune evasion literature over the past 20 years has highlighted that many bacterial pathogens express molecules that allow them to escape detection by activating receptors (Avondt, Sorge, and Meyaard 2015). For example, *Salmonella typhimurium* modification of the lipid A component of LPS reduces TLR4 activation and NF-κB production (Kawasaki, Ernst, and Miller 2004). More recently it has emerged that bacterial pathogens exploit inhibitory receptors and their signalling for immune evasion purposes. Several distinct mechanisms have been described in a recent review by (Avondt, Sorge, and Meyaard 2015): (1) Direct binding of bacteria or bacterial molecules to inhibitory receptors. Engagement of inhibitory receptors induces ITIM phosphorylation that promotes inhibitory signalling and suppresses immune cell activity (Slevogt et al. 2008) (2) Low-affinity binding of bacteria or their molecules to activating receptors. Low avidity binding to certain ITAM receptors causes recruitment of SHP-1/2 phosphatases, promoting inhibitory signalling, thereby increasing bacterial resistance to host clearance (Pinheiro da Silva et al. 2007) (3) Translocation of bacterial ITIM effector proteins into the host cell cytoplasm. This serves to promote inhibitory signalling and suppress proinflammatory responses such as the secretion of cytokines (Yan et al. 2012) (4) Insertion of ITIM-bearing receptors into cell membranes. This effect increases the activation threshold of immune cells, thereby suppressing immune cell activation and effector functions (5) Expression of effector proteins that dephosphorylate molecules involved in activating signalling cascades. This results in suppression of activating signalling and suppression of immune cell activation (Choi et al. 2013).

Of these mechanisms, the direct binding of bacteria or their molecules to inhibitory receptors is the best characterised. This likely represents a key mechanism that bacteria utilise for immune evasion because inhibitory receptors are a) surface exposed and readily available to bind, b) expressed by the key immune cells involved in bacterial clearance c) control a range of immune cell effector functions. Therefore, through direct binding of inhibitory receptors, bacteria likely greatly enhance their immune evasion and virulence potential. Thus, a large amount of research has focused on identifying the bacterial ligands that bind inhibitory receptors as they likely represent important factors driving invasive disease and are likely good targets of antibacterial therapeutics.

1.5.1 Direct interaction of human bacterial pathogens with inhibitory receptors

Numerous bacteria are known to directly interact with inhibitory receptors including *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Helicobacter pylori*, and *Escherichia coli* (Avondt, Sorge, and Meyaard 2015). To determine the role of these interactions in immune evasion, it is first necessary to identify the bacterial ligands involved. Several bacterial ligands have been identified that bind inhibitory receptors of the Siglec family and CEACAM1 (**Table 1.3**). Additionally, initial work suggests that *S. aureus* interacts with SIRL-1 through the release of α -phenol soluble modulins (α -PSMs).

The first interaction of a human bacterial pathogen with an inhibitory receptor was the binding of Opa proteins from *Neisseria spp.* to CEACAM1. Since then, CEACAM1 has been identified as the target of ligands expressed by a range of different human bacterial pathogens. These include β protein, expressed by *S. agalactiae*; R28, expressed by *S. pyogenes*; ubiquitous surface protein A1 (UspA1), expressed by *Moraxella catarrhalis*; outer membrane protein P1 (OMP P1), expressed by *Haemophilus influenzae*; Afa/Dr-I subfamily of adhesins (Dr, F1845, and AfaE-III) expressed by *E. coli*; HopQ expressed by *H. pylori*; CEACAM binding protein (CbpF) expressed by *Fusobacterium spp* (**Table 1.3**).

Inhibitory Receptors	Human Bacterial Pathogens	Human Pathogen Ligands	REFERENCES
Siglec-11	<i>E. coli</i>	polysialic acid capsule	(Schwarz et al. 2017)
Siglec-9	<i>S. agalactiae</i>	Sialyated capsular polysaccharide	(Carlin, Uchiyama, et al. 2009)
Siglec-9	<i>P. aeruginosa</i>	Adsorbed sialic acids	(Khatua, Bhattacharya, and Mandal 2012)
Siglec-9	<i>S. pyogenes</i>	Hyaluronic capsule	(Secundino et al. 2016)
Siglec-7	<i>S. agalactiae</i>	β protein	(Fong et al. 2018)
Siglec-5	<i>S. agalactiae</i>	β protein	(S. R. Ali et al. 2014)

CEACAM1	<i>N. gonorrhoeae</i>	Opa	(Sadarangani, Pollard, and Gray-Owen 2011)
CEACAM1	<i>N. meningitidis</i>	Opa	(Kuespert, Roth, and Hauck 2011)
CEACAM1	<i>H. influenzae</i>	OMP	(Kim et al. 2019)
CEACAM1	<i>E. coli</i>	Afa/Dr family of adhesions	(Berger et al. 2004)
CEACAM1	<i>H. pylori</i>	HopQ	(Hamway et al. 2020)
CEACAM1	<i>S. agalactiae</i>	β protein	(van Sorge et al. 2021)
CEACAM1	<i>S. pyogenes</i>	R28	(Catton et al. 2023)
CEACAM1	<i>M. catarrhalis</i>	Ubiquitous surface protein (UspA1)	(Slevogt et al. 2008)
CEACAM1	<i>H. pylori</i>	HopQ	(Javaheri et al. 2016)
CEACAM1	<i>Fusobacterium spp.</i>	CbpF	(Brewer et al. 2019)
SIRL-1	<i>S. aureus</i>	α phenol-soluble modulins	(Rumpret et al. 2021)

Table 1.3 Bacterial ligands that interact with inhibitory immune receptors.

Interestingly, several of these bacterial pathogens have evolved CEACAM1 binding ligands that are structurally distinct. For example, OMP P1 adopts an eight-stranded β barrel structure, whilst HopQ adopts an α - β folded structure (Kim et al. 2019). The presence of structurally unrelated bacterial ligands for CEACAM1 suggests convergent evolution of ligands that bind inhibitory receptors and confer survival advantages to pathogens. Alternatively, proteins from different species contain structurally related domains that confer CEACAM1 binding; for example, β protein and R28 of *S. agalactiae* and *S. pyogenes*, respectively, share a structurally related IgI3 fold that allows interaction with CEACAM1 (van Sorge et al. 2021). In addition, *Acinetobacter baumannii* interacts with CEACAM1 through an unknown ligand (Ambrosi et al. 2020). Collectively, these data highlight that many bacterial pathogens have specifically evolved mechanisms to directly interact with the inhibitory immune receptor CEACAM1.

The inhibitory Siglec receptors have also emerged as a common target of bacterial pathogens. Siglec receptor interactions have been discovered for *S. agalactiae*, *S. pyogenes*, *P. aeruginosa*, and *E. coli* (**Table 1.3**). Several of these bacteria express a capsule that is recognised by Siglec receptors: *E. coli* expresses a polysialic acid capsule that is recognised by Siglec-11; *S. agalactiae* expresses a polysaccharide capsule that is recognised by Siglec-9; *S. pyogenes* expresses a hyaluronic capsule that binds Siglec-9 (Schwartz et al. 2019; Carlin, Chang, et al. 2009; Secundino et al. 2016). As an example of an interaction in further detail, Siglec-9 directly binds Sia α 2-3Gal β 1-4GlcNAc units that are on terminal ends of glycans present in *S. agalactiae* polysaccharide capsule (Carlin, Chang, et al. 2009). The same modifications are also often present in human glycan structures. Thus, *S. agalactiae* uses molecular mimicry to interact with Siglec-9 and subvert immune responses. Importantly, *S. agalactiae* also targets Siglec receptors through an additional distinct mechanism. *S. agalactiae* directly interacts with Siglec-5 and Siglec-7 via the expression of a surface protein called β protein (Ali et al. 2014; Fong et al. 2018). Fong et al went on to characterise the Siglec-7- β interaction, showing that Siglec-7 express an amino-terminal V-set domain that directly interacts with the N-terminal domain of β protein (B6N) (Fong et al. 2018).

More recently, *S. aureus* has been shown to secrete factors that engage the inhibitory receptor SIRT-1. SIRT-1 senses host damage and suppresses immune activation in response to limit damage. Recent work has demonstrated that SIRT-1 functions to suppresses neutrophil activation in response to S100 damage associate molecules (Rumpret et al. 2021; 2021). *S. aureus* subverts this immune homeostatic mechanism by secreting α -PSMs that also activate SIRT-1. α -PSMs are peptides secreted by almost all staphylococci species that are cytotoxic to human cells. Utilising SIRT-1 receptor reporter cell lines demonstrated that α -PSMs activate SIRT-1 (Rumpret et al. 2021; 2021). Thus, *S. aureus* secrete α -PSMs to target SIRT-1 and utilise a host mechanism that normally serves to limit host damage.

1.5.2 Functional impact of inhibitory receptor and bacteria interactions

Inhibitory receptors are thought to be directly targeted by bacteria and other pathogens to modulate immune responses and promote pathogen survival. Bacterial ligands that induce co-ligation of inhibitory and activating receptors can suppress activating signals, immune cell activity, and immune effector functions (Avondt, Sorge, and Meyaard 2015).

CEACAM1 represents a model receptor for understanding the functional consequences of bacterial interactions with inhibitory receptors. Research has shown that CEACAM1 ligation by bacterial ligands suppresses immune responses and promotes bacterial invasion. For example, *M. catarrhalis* which express UspA1 can bind to CEACAM1 and inhibit TLR-2 signalling (Slevogt et al. 2008). This CEACAM1-mediated suppression of TLR-2 signalling reduces NF- κ B-dependent release of proinflammatory cytokines such as IL-8 in pulmonary epithelial cells (Slevogt et al. 2008). As IL-8 is a neutrophil chemotactic factor, UspA1-CEACAM1 interactions likely function to suppress neutrophil recruitment and promote bacterial survival. Likewise, *N. gonorrhoeae* express the Opa protein that also binds CEACAM1 to promote immune evasion (Slevogt et al. 2008). Interestingly, there are several CEACAM1-mediated immune evasion mechanisms that remain to be elucidated. For example, studies

are required to assess the functional impact of *S. agalactiae* targeting CEACAM1 by β protein (van Sorge et al. 2021). Furthermore, the mechanism through which *A. baumannii* targets CEACAM1 has not been elucidated with the bacterial ligand still to be identified.

Investigations into inhibitory Siglec have also shed light on the functional effects of bacterial targeting inhibitory receptors. There are several examples of Siglec engagement that suppress multiple immune cells and their effector functions. *S. agalactiae* express a capsular polysaccharide that binds Siglec-9 on neutrophils suppressing ROS production and NET release resulting in increased bacterial survival (Secundino et al. 2016). *S. agalactiae* also expresses the surface β protein that binds Siglec-5 and Siglec-7 suppressing ROS production, NETosis and phagocytosis (Fong et al. 2018; Carlin, Chang, et al. 2009). These properties promoted *S. agalactiae* survival in blood. In addition, *S. pyogenes* expresses a capsule that binds Siglec-9 blocking neutrophil NETosis and ROS production promoting bacterial survival (Secundino et al. 2016). Finally, *E. coli* express a polysaccharide capsule that binds Siglec-11 promoting bacterial survival, likely through suppression of macrophage activation (Schwarz et al. 2017). Collectively, these studies demonstrate that bacterial pathogens target inhibitory Siglec to promote immune evasion and enhance bacterial survival.

1.6 Human bacterial pathogens target inhibitory LILR

1.6.1 Pathogen ligands of inhibitory LILRs

The inhibitory LILRs have also been identified as forming interactions with human bacterial pathogens. However, unlike the bacteria-CEACAM1 or bacteria-Siglec interactions, the bacterial ligands for these interactions have yet to be defined. The first study to detect interactions screened pathogenic bacteria for their interaction with LILRB1 and LILRB3 expressing cells (Nakayama et al. 2007). This study revealed that LILRB1-expressing cells interact with *S. aureus*, *E. coli*, and *H. pylori* (Nakayama et al. 2007). Furthermore, LILRB3-expressing cells interact with *S. aureus* and *H. pylori* (Nakayama et al. 2007). Data suggest that human bacterial pathogens target inhibitory LILR. However, the ligands remain to be elucidated and the functional consequences of these interactions remain unknown.

Whilst bacterial ligands for inhibitory LILR have yet to be identified, several protozoan and viral pathogen ligands have been identified that interact with inhibitory LILR (**Fig 1.10**). LILRB1 binds to pathogen-derived molecules such as UL18 from human cytomegalovirus (HCMV) (Chapman, Heikeman, and Bjorkman 1999); E4, D39, D51, C1, and R1 β ligands from human adenovirus (Martinez-Martin et al. 2016); and 7 repetitive interspersed families of polypeptides (RIFIN) proteins (PF3D7_1373400, PF3D7_0937700, PF3D7_1041200, PF3D7_0632400, PF3D7_0324800, PF3D7_1041000 and PF3D7_1300700) expressed by *Plasmodium falciparum* (Saito et al. 2017). LILRB2 also binds to RIFIN (PfKH02_07001600) derived from *P. falciparum*. Of note, in contrast to host ligands

that generally bind LILRB1 and LILRB2 D1-D2 domains, RIFIN molecules bind the D3-D4 domains of LILRB1 or LILRB2 (Chen et al. 2021; Sakoguchi et al. 2021).

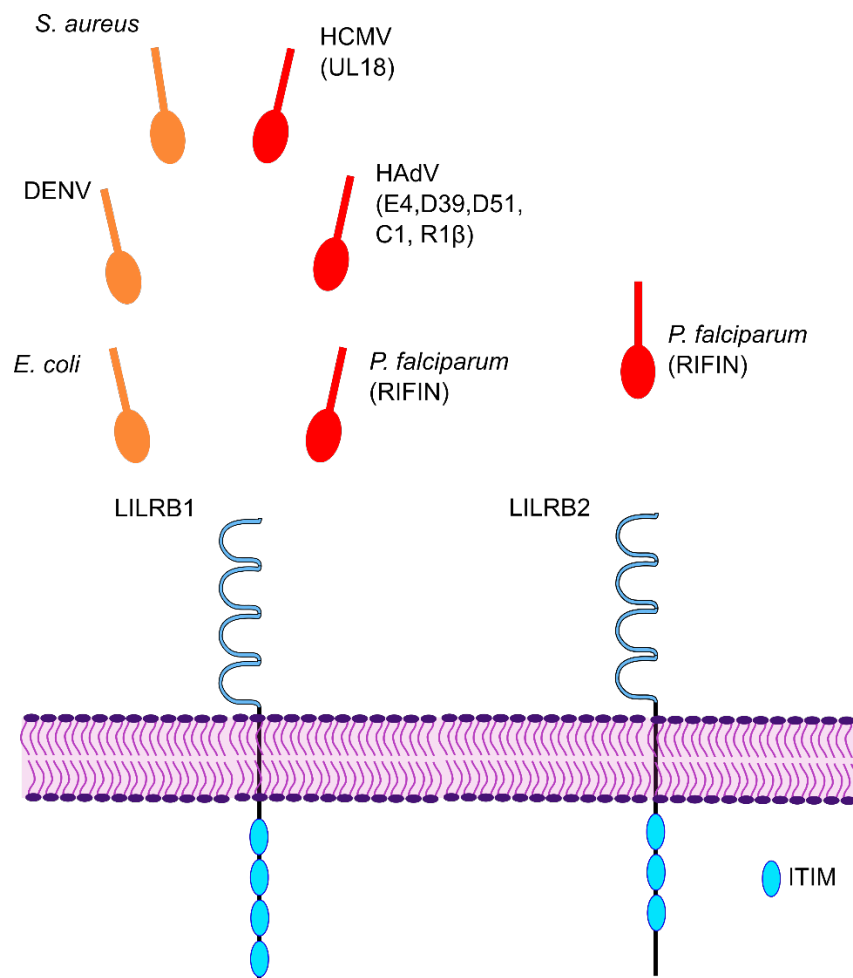


Figure 1.10. Inhibitory LILR interactions with pathogens. LILRB1 and LILRB2 are the only inhibitory LILR that are currently known to bind pathogens; however, not all the pathogen ligands have been identified. The schematic shows pathogens with ligands identified coloured in red, whilst those with ligands yet to be determined are coloured in orange (*S. aureus*, Dengue Virus (DENV), and *E. coli*). LILRB1 binds: UL18, from human cytomegalovirus (HCMV) (Chapman, Heikeman, and Bjorkman 1999); E4, D39, D51, C1 and R1β ligands from human adenovirus (HAdV) (Martinez-Martin et al. 2016); and 7 repetitive interspersed families of polypeptides (RIFIN) proteins (PF3D7_1373400, PF3D7_0937700, PF3D7_1041200, PF3D7_0632400, PF3D7_0324800, PF3D7_1041000 and PF3D7_1300700) expressed by *P. falciparum* (Saito et al. 2017). RIFIN (PfKH02_07001600) derived from *P. falciparum* also binds LILRB2.

1.6.2 Functional consequences of inhibitory LILR and pathogen interactions

Since no bacterial ligands have been defined for inhibitory LILRs, there are currently no studies investigating the impact of targeting inhibitory LILRs in the context of bacterial infection. However, studies investigating viral and protozoan interactions point to a role in immune evasion. Of the pathogens that interact with inhibitory LILR, two have been demonstrated to enhance immune evasion: LILRB1-RIFIN (*P. falciparum*) and LILRB1-UL18 (HCMV).

P. falciparum, a pathogenic protozoan responsible for causing life-threatening malaria, interacts with both LILRB1 and LILRB2. During pathogenesis, *P. falciparum* invades erythrocytes inducing the expression of a variety of RIFIN proteins on erythrocyte cell surface that promote immune evasion although the precise mechanisms involved are not well elucidated (Bachmann et al. 2015). However, RIFIN binding to LILRB1 has been shown to suppress NK cell activation thereby providing a mechanism for RIFIN-mediated immune evasion (Chen et al. 2021).

The second example of pathogens targeting LILRB1 for immune evasion is by HCMV. Although HCMV carriage is asymptomatic, due to its high seroprevalence of ~60-90% it is the leading cause of congenital infections and can cause serious infections in immunocompromised individuals. NK cells are essential for recognising and lysing HCMV-infected cells to clear infection (Fulkerson et al. 2021). HCMV expresses UL18, a homolog of MHC class I antigens, that binds LILRB1 and is proposed to suppress NK cell activation and promote immune evasion (Cosman et al. 1997). This model is supported by association studies in humans demonstrating that LILRB1 polymorphisms in the HCMV binding domain alter LILRB1-UL18 binding and increase susceptibility to HCMV posttransplant (Yu et al. 2018).

Similar to bacterial pathogens, some viral pathogens are known to interact with inhibitory LILRs through undefined molecular mechanisms. For example, the DENV virus binds LILRB1 through a yet-to-be-identified ligand. Whilst the ligand is unknown, functional studies have shown that DENV interaction with LILRB1 promotes immune evasion by suppressing FcγR-mediated expression of type-I interferon-stimulated genes and inhibiting trafficking of DENV to lysosomes (Chan et al. 2014; Ong et al. 2017). Likewise, several human adenovirus (HAdV) ligands were identified to interact with LILRB1 using a large-scale protein interaction screen against 1,500 human proteins. However, the functional consequences of HAdV-LILRB1 interactions for immune evasion are unknown (Martinez-Martin et al. 2016).

Collectively, these studies demonstrate that pathogens target inhibitory LILRs likely to promote immune evasion. It also highlights that additional research is required to identify the bacterial pathogen ligands and determine whether bacteria engage inhibitory LILRs for immune evasion purposes.

1.7 Streptococcal pathogens

Streptococcal pathogens are Gram-positive bacteria that are a major source of global health burden causing a range of diseases. *Streptococcus pyogenes* (also referred to as, Group A *Streptococcus*, GAS), *Streptococcus*

agalactiae (also referred to as, Group B *Streptococcus*, GBS) and *Streptococcus pneumoniae* are common streptococcal pathogens which contribute significantly to morbidity and mortality worldwide. Over a third of the bacterial ligands identified (6/17) that bind to human inhibitory receptors are derived from *S. agalactiae* and *S. pyogenes* (Table 1.3). This suggests that the targeting of inhibitory receptors is a key immune evasion strategy for streptococcal pathogens.

1.7.1 *Streptococcus agalactiae*

1.7.1.1 Epidemiology

S. agalactiae, also referred to by the Lancefield classification of Group B *Streptococcus* (GBS), is generally part of a healthy human microbiota in the gastrointestinal and urogenital tracts of ~ 30% of the human population. However, *S. agalactiae* is also an opportunistic pathogen and vertical transmission of *S. agalactiae* from pregnant mothers to their neonates can lead to neonatal invasive diseases such as sepsis, pneumonia, and meningitis (Yu et al. 2018). As such, *S. agalactiae* is predominantly known as a neonatal pathogen that causes an estimated 409,000 infections in infants annually resulting in ~150,000 deaths (Seale et al. 2017; Aiweesakun et al. 2022). Neonatal diseases are divided into early onset (EOD), occurring at <7 days of life, and late onset disease (LOD), between 7-90 days of life. The incidence of invasive *S. agalactiae* in infants (0-89 days) worldwide is 0.49 per 1000 live births, with the highest rates in Africa at 1.12 and the lowest in Asia (0.30). Introduction in 1973 of intrapartum antibiotic prophylaxis (administration of antibiotics prior to birth) has reduced EOD neonatal disease by ~80%; however, LOD has remained the same (Schrag et al. 2016). In addition, *S. agalactiae* also causes diseases in pregnant women and non-pregnant adults, particularly in elderly populations (Skoff et al. 2009). There are currently no vaccines against *S. agalactiae* to provide protective immunity in at-risk individuals (Loh et al. 2021; Johri et al. 2006). Furthermore, the rise of clindamycin-resistant *S. agalactiae* threatens our capacity to effectively treat *S. agalactiae* infections (Huang et al. 2016; Belard et al. 2015).

Certain *S. agalactiae* serotypes and genotypes are associated with invasive disease. Historically, *S. agalactiae* has been classified into 10 serotypes based on the capsular polysaccharide – Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX. Long-term epidemiological studies suggest that serotypes Ia, Ib, II, III and V are associated with invasive disease (Francois Watkins et al. 2019; Slotved and Hoffmann 2020; Hsu et al. 2022; Bobadilla et al. 2021). Serotype III is the dominant serotype causing invasive neonatal disease worldwide (Lin et al. 2006; Ip et al. 2016; Madrid et al. 2017). The availability of *S. agalactiae* genome sequences over the past 15-20 years has led to improved knowledge of the global *S. agalactiae* population structure and the identification of virulent clones (McGee et al. 2021; Aiweesakun et al. 2022; Da Cunha et al. 2014). Remarkably the majority of carriage and clinical invasive isolates cluster into just 5 major clonal complex (CC) lineages: CC1, CC10, CC17, CC19 and CC23 (Feil et al. 2004; Da Cunha et al. 2014). Nationwide surveillance studies have also demonstrated that specific CC are

associated with diseases, such as CC17-A1 with infant meningitis (Kassel et al. 2021). Furthermore, specific clones such as serotype III ST-17 are hypervirulent being more prevalent in invasive disease compared with carriage (91% versus 33%) (Lin et al. 2006).

1.7.1.2 Pathogenesis

S. agalactiae must overcome several barriers to cause invasive diseases such as bacteraemia and meningitis. The female rectovaginal tract is the main host colonisation niche, which is required for *S. agalactiae* transmission to the foetus or infant and subsequent infection. To cause invasive diseases *S. agalactiae* must successfully colonise the female reproductive tract or rectum, cause an ascending infection, be transmitted from mother-to-infant, translocate across epithelial barriers, evade innate immune responses, and invade deeper tissue. *S. agalactiae* can spread *in utero* by ascending infection or during birth by aspiration of amniotic fluid (Sitkiewicz et al. 2009).

The capacity of *S. agalactiae* to cause invasive diseases is through the expression of key virulence factors. These virulence factors perform key functions such as promoting adhesion, acting as toxins to kill host cells, and evading immune responses that contribute to invasive disease. Several studies have investigated the functional consequences of specific virulence factors and their role in causing invasive disease. For example, C5a peptidase (ScpB) proteolytically cleaves C5a anaphylaxin thereby inhibiting neutrophil recruitment and enhance bacterial adhesion through binding fibronectin (Cheng et al. 2002). Thus, ScpB facilitates the pathogenesis of invasive diseases by evading immune responses and promoting adhesion. Indeed, ScpB expression is associated with bacteraemia and pneumonia (López et al. 2018). Another example virulence factor is hypervirulent adhesin (HvgA). Once *S. agalactiae* enters the bloodstream it can translocate to the blood-brain barrier causing meningitis. Indeed, expression of HvgA is necessary to induce meningitis in mice by increasing *S. agalactiae* binding to choroid plexus epithelial and microvascular endothelial cells that make up the blood-brain barrier (Tazi et al. 2010). There are numerous other bacterial factors that have been identified and have broad functions that are important for *S. agalactiae* pathogenesis - reviewed in detail in (Y. Liu and Liu 2022), (Rajagopal 2009), and (Pietrocola et al. 2018). Importantly, *S. agalactiae* express capsule and β protein virulence factors that are known to interact with inhibitory receptors and will be reviewed in detail below.

Capsular Polysaccharide – immune protection and targeting inhibitory receptors

The capsule is a highly versatile virulence factor that encases *S. agalactiae* and interacts with host factors to promote immune evasion. The capsule polysaccharide capsule is composed of polymers consisting of sialic acid, glucose, galactose, and *N*-acetylglucosamine that encase *S. agalactiae* (Spellerberg 2000). The exceptions are capsule polysaccharide from serotype VI which lacks *N*-acetylglucosamine, and serotype VIII which contains rhamnose but not *N*-acetylglucosamine (Spellerberg 2000). Capsule polysaccharide promotes *S. agalactiae*

immune evasion and virulence through several mechanisms. Firstly, sialic acid expressed on the terminal sugar side chain mimics host glycan sugars, and allows *S. agalactiae* to escape from immune surveillance (Carlin, Uchiyama, et al. 2009). As discussed in Section 1.5.1, the “mimic” sialic acid on the surface of *S. agalactiae* is detected by the inhibitory receptor Siglec-9, which suppresses the immune cell effector responses (Carlin, Uchiyama, et al. 2009; Chang et al. 2014). Secondly, capsular polysaccharide inhibits the deposition of the complement opsonin C3b on the *S. agalactiae* cell surface, which likely inhibits complement activation and recognition by immune cells (Marques et al. 1992). As complement receptors CR1 and CR3 recognise and phagocytose C3b opsonised microbes (Boero et al. 2023), the reduced C3b deposition suppresses complement-mediated phagocytosis and killing. Indeed, isogenic capsule mutants of *S. agalactiae* have increased susceptibility to opsonophagocytic killing by neutrophils (Marques et al. 1992). Thus, the capsule promotes immune evasion by targeting immune inhibitory receptors and complement factors to inhibit immune cells and effector responses.

β protein – a surface anchored protein that targets multiple inhibitory immune receptors

β protein is a cell wall-associated protein that binds to several components of the human immune system. The β protein was first identified as one component of the C protein antigen complex, along with α protein (Wilkinson and Eagon 1971; Bevanger and Maeland 1979). The *bac* gene encodes for the β protein and carriage is predominantly associated with virulent serotypes Ia, Ib, and II; to a lesser degree, serotypes III, V, VI, and IX (Bobadilla et al. 2021; Kong et al. 2006; Manning et al. 2006; Dore et al. 2003).

The β protein suppresses neutrophil effector functions to promote immune evasion. There are several potential mechanisms through which β protein mediates immune evasion. This is because β protein interacts with many components of the human immune system, including IgA, FH, Siglec-5, Siglec-7, Siglec-14, CEACAM1 and CEACAM5 (Carlin et al. 2009; Ali et al. 2014; Fong et al. 2018; van Sorge et al. 2021; Jerlström, Chhatwal, and Timrnis 1991). The first ligands to be identified for β protein were the soluble factors FH and IgA (Lindahl et al. 1990; Areschoug et al. 2002). The binding of these immune components may serve to evade immune surveillance: β protein non-opsonically binds IgA via the Fc region, whilst sequestering FH to bacterial surfaces inhibits C3b deposition which is important opsonisation (Areschoug et al. 2002).

Additionally, β protein was shown to interact with the inhibitory receptor Siglec-5 (Carlin et al. 2009). Binding β to this inhibitory receptor was suggested as a mechanism for dampening immune cell responses. Indeed, β protein induces inhibitory signalling via Siglec-5 (Carlin et al. 2009). This study also revealed that neutrophil activity and effector functions were enhanced in response to *S. agalactiae* isogenic knockout *bac* strains, and these mutant strains were more susceptible to neutrophil killing (Carlin, Chang, et al. 2009). Thus, the β protein is important for immune evasion by suppressing neutrophil effector functions. However, it was recently revealed that β protein also binds the inhibitory receptor CEACAM1 (van Sorge et al. 2021). The binding of β protein to CEACAM1 is likely to have a similar function to the binding of β protein to Siglec-5 as both induce inhibitory

signals through ITIM domains. However, inhibitory signalling assays have yet to be performed (van Sorge et al. 2021). Whether these β -mediated interactions work synergistically, exhibit redundancy, or are context-dependent is still unclear. Thus, whilst β protein expression is clearly important for immune evasion, the precise mechanism(s) and interactions that drive the immune evasion process remain to be elucidated.

1.7.2 *Streptococcus pyogenes*

1.7.2.1 Epidemiology

S. pyogenes (Group A *Streptococcus*; GAS) is a significant global public health burden that causes a plethora of diseases from mild infections to severe invasive diseases and immune disorders. *S. pyogenes* is a human-specific bacterial pathogen that lists among the top 10 causes of human mortality from infectious disease with at least 18.1 million severe cases recorded in 2005, resulting in at least 517, 000 deaths per year (Carapetis et al. 2005). *S. pyogenes* predominantly colonises the throat and skin causing superficial diseases such as pharyngitis, scarlet fever, and impetigo; however, these infections can lead to more serious life-threatening invasive diseases such as necrotising fasciitis, streptococcal toxic shock syndrome (STSS), puerperal sepsis, and bacteraemia (Brouwer et al. 2023). Furthermore, repeated or untreated *S. pyogenes* infections can lead to life-threatening autoimmune sequelae such as acute rheumatic fever, rheumatic heart disease, and post-streptococcal glomerulonephritis (Walker et al. 2014). There is currently no vaccine available, and whilst *S. pyogenes* has remained susceptible to antibiotics such as penicillin, there is a rise in antibiotic resistance (Johnson and LaRock 2021).

Contemporary severe invasive *S. pyogenes* infections are disproportionately caused by specific serotype/genotypes in developed nations. *S. pyogenes* is classified serologically and genotypically based on M protein and its encoding gene *emm*. Due to the hypervariability of the N-terminal domain in M proteins, there are >250 different serotypes and genotypes. Invasive infections in the developed world are often associated with genotypes *emm1*, *emm3*, *emm12*, *emm28*, and *emm89* (Steer et al. 2009). In contrast, *S. pyogenes* populations in developing nations, such as those in Africa and the Pacific, exhibit a higher diversity of *emm* types and have no dominant *emm* type that causes invasive infections (Steer et al. 2009). The disease burden of *S. pyogenes* is highest in developing nations. Though the reasons for this are unclear, it is possible that higher genotype diversity may be a contributing factor, as well as socioeconomic status (Brouwer et al. 2023). Outbreaks of *S. pyogenes* infections also occur and appear to be caused by specific genotypes and sub-lineages. For example, several studies have identified the *emm1* genotype as being associated with invasive disease outbreaks (Feng et al. 2016). More recently, a sub-lineage of M1, called M1_{UK}, has been associated with an increase in superficial and invasive diseases (Lynskey et al. 2019; Li et al. 2020).

1.7.2.2 Pathogenesis

S. pyogenes is perfectly adapted to colonise humans as the only known biological reservoir. The main sites of colonisation are the nasopharyngeal tract and skin through which transmission to new hosts via respiratory drops or direct contact occurs (Bessen, Smeesters, and Beall 2018). *S. pyogenes* cause a broad range of diseases in humans that likely require the expression of a broad range of virulence factors. Similar to *S. agalactiae*, these virulence factors likely function to promote transmission, colonisation, invasion and immune evasion. The human-restricted nature of *S. pyogenes* limits the reliability of *in vivo* models for studying *S. pyogenes* pathogenesis. However, there are transgenic models and more recently the development of a controlled human infection model for studying *S. pyogenes* causing pharyngitis (Osowicki et al. 2021).

To cause invasive disease, *S. pyogenes* likely utilises both intracellular and paracellular mechanisms to translocate across epithelial barriers and gain access to the bloodstream. Several virulence factors target epithelial integrity providing mechanisms for invasion. For example, *S. pyogenes* expresses a capsule composed of hyaluronic acid that binds the cell surface adhesin CD44 on keratinocyte cells (Cywes and Wessels 2001). These interactions disrupt intercellular junctions promoting paracellular tissue invasion (Cywes and Wessels 2001). *S. pyogenes* may also cross epithelial barriers by invading epithelial cells. This is achieved when *S. pyogenes* express invasins such as *S. pyogenes* fibronectin-binding protein (Sfbl) that promote the internalisation of the bacterium into epithelial cells (Molinari et al. 1997). Once internalised, *S. pyogenes* can secrete streptolysin O (SLO) and NAD glycohydrolase (NADase) promoting cell death and proinflammatory responses (Flaherty et al. 2015). Pro-inflammatory responses, together with the secretion of toxins such as streptococcal pyrogenic exotoxin B (SpeB), further damage epithelial cell wall integrity and provide access for *S. pyogenes* to deeper tissue that is necessary to cause invasive disease. Throughout pathogenesis *S. pyogenes* must evade immune responses, and several of these virulence factors serve an additional function of promoting immune evasion. This includes capsule, surface proteins, and secreted factors reviewed in detail in (Smeesters, McMillan, and Sriprakash 2010; Brouwer et al. 2023; Walker et al. 2014).

M protein – a surface anchored protein with immune evasion properties

M and/or M-like proteins are major virulence factors expressed by almost all *S. pyogenes* invasive strains. The M protein is a dimeric coiled-coil fibrillar protein that is diverse with over 250 variants being identified that each correspond to a unique serotype (Russo et al. 2020). The M protein variant is denoted by the *emm* type; for example, M1 protein is encoded by the *emm1* gene. The C terminal domain is highly conserved and covalently attached to the cell wall via sortase cleavage of a LPxTG motif (Russo et al. 2020). The N terminal domain is hypervariable, with the first 30 codons defining the serotype (Bessen, Smeesters, and Beall 2018). M proteins interact with several host factors including antibodies IgG and IgA; complement regulating factors FH, FHL-1, and C4BP; plasma proteins plasminogen, fibrinogen, and albumin; and antimicrobial peptide cathelicidin (Smeesters, McMillan, and Sriprakash 2010). Through these interactions, M proteins are proposed to facilitate immune evasion.

Firstly, the M protein evades immune surveillance by phagocytes through non-opsonically binding IgA and IgG antibodies (Stenberg, O'Toole, and Lindahl 1992). IgA and IgG are important for opsonising microbes for recognition by immune cells, thus, evasion of antibody opsonisation promotes *S. pyogenes* survival in the human body and promotes invasive diseases. Indeed, Lindahl et al showed that the non-opsonic binding of IgA to M22 promoted bacterial survival in human whole blood (Carlsson et al. 2003). Furthermore, multiple M proteins including M22, M28, and M12 bind all subclasses of IgG antibody (IgG1-IgG4) and these interactions likely promote bacterial survival in blood (Smeesters, McMillan, and Sriprakash 2010).

Secondly, M protein suppresses complement activation to enhance immune evasion. Several M proteins sequester complement-regulating proteins such as FH and C4BP thereby inhibiting complement activation (Smeesters, McMillan, and Sriprakash 2010). For example, M22 binds C4BP resulting in deposition of C3b (C3d) and C4b (C4c and C4d) fragments suppressing complement activation (Carlsson et al. 2003). Likewise, the M1 protein binds both C4BP as well as FH to inhibit C4b and C3b deposition (Smeesters, McMillan, and Sriprakash 2010; Horstmann et al. 1988).

Finally, M proteins directly and indirectly inhibit phagocyte effector functions for immune evasion. M proteins directly lyse phagocytes by inducing programmed cell death. Soluble M1 protein has been shown to activate the NLRP3 inflammasome inducing pyroptotic cell programmed death in macrophages (Valderrama and Nizet 2018). M proteins also indirectly inhibit phagocyte effector functions by neutralising antimicrobial peptides (LaRock et al. 2015). Antimicrobial peptides such as cathelicidin (LL-37) are secreted by neutrophils and macrophages to disrupt and damage microbial cell surfaces and promote inflammation (Kościuczuk et al. 2012). M1 protein suppresses neutrophil translocation, killing and NETosis by neutralising cathelicidin (LaRock et al. 2015). Thus, M proteins suppress phagocyte effector functions through multiple mechanisms.

Streptococcal pyrogenic exotoxin B (SpeB) - soluble immune evasion molecule

S. pyogenes not only directly target host factors through surface-bound virulence factors but also through secreted toxins and proteases. One such protease is SpeB. The *speB* gene is carried by all *S. pyogenes* strains (Hollands et al. 2008) and encodes a broad-spectrum cysteine protease that promotes immune evasion through proteolytically cleaving several host factors. SpeB is important for establishing both superficial and invasive diseases (Svensson et al. 2000; Kuo et al. 1998; Hollands et al. 2008). Indeed, Δ *speB* *S. pyogenes* strains exhibit significantly reduced virulence in a mouse model of systemic infection (Hollands et al. 2008).

SpeB targets several host factors to promote immune evasion. Firstly, SpeB degrades all antibody subtypes with IgG, IgA, IgM and IgD antibodies being cleaved into Fc and Fab fragments, whilst IgE is completely degraded (Collin and Olsén 2001). Degradation of antibodies inhibits immune cell recognition and classical complement pathway activation. SpeB also inhibits alternative complement pathway activation by degrading C3b (Terao 2012). Through the degradation of antibody and complement opsonins, SpeB enhance evasion of phagocytic

immune cells. In addition to inhibiting phagocytosis, SpeB enhances *S. pyogenes* immune evasion by degrading antimicrobial peptides such as LL-37 (Schmidtchen et al. 2002; Schwäbe et al. 2022). Finally, SpeB degrades a huge range of chemokines such as CXCL1-CXCL7 and CXCL10-CXCL16 (Egesten et al. 2009). Through these interactions, SpeB suppresses immune cell recruitment and proinflammatory functions. Indeed, a streptococcal toxic shock syndrome (STSS) mouse model demonstrated that SpeB inhibits neutrophil and promotes *S. pyogenes* survival in whole blood (Terao 2012). Thus, SpeB is a model virulence factor that interacts with a plethora of host factors enabling immune evasion and bacterial survival.

1.7.3 *Streptococcus pneumoniae*

1.7.3.1 Epidemiology

S. pneumoniae is one of the 12 priority pathogens determined by WHO in 2017 and is a major global health burden (Weiser, Ferreira, and Paton 2018). It is the most common cause of community-acquired pneumonia and the major cause of mortality in children less than 5 years old, with recent estimates of 294,000 deaths in HIV-uninfected children in 2015 (Wahl et al. 2018). The upper respiratory tract is the main ecological niche and rates of colonisation are as high as 60% in children (Yahiaoui et al. 2016). Factors that affect colonisation include age, socioeconomic status, geography and genetic background. In addition to causing pneumonia, *S. pneumoniae* causes other serious invasive diseases such as bacteraemia, meningitis, and otitis media. The mortality rates attributable to *S. pneumoniae* are much higher in African and Southeast Asian countries, compared with Western nations (Wahl et al. 2018). There are several commercially available pneumococcal vaccines based on the polysaccharide that encapsules *S. pneumoniae* which have reduced the incidence of pneumococcal diseases; however, the genetic promiscuity of *S. pneumoniae* has resulted in vaccine escape reducing the effectiveness of current vaccines (Johnson et al. 2023).

Almost all *S. pneumoniae* strains express a polysaccharide capsule that determines the serotype. There are over 100 serotypes identified to date (Ganaie et al. 2020). However, ~75% of isolates that cause invasive disease in children are from 12 different serotypes: 14, 4, 1, 6A, 6B, 3, 8, 7F, 23F, 18C, 19F, and 9V (Lynch and Zhanel 2009). These serotypes are not distributed equally worldwide as certain serotypes are more prevalent in particular geographical regions. For example, in China serotypes 23F, 19A/F, 3, and 14 are the most prevalent, whilst in Ethiopia serotypes 15A/B/C and 6A are the most prevalent (Zhou et al. 2022; Sharew et al. 2021). Strains are also characterised into sequence types (ST) or clones based on genome sequences. Certain *S. pneumoniae* clones are associated with invasive disease. For example, a comparison of 150 invasive and 351 carriage *S. pneumoniae* strains isolated from children in the UK revealed that only three ST types (ST9, ST124 and ST113) were highly associated with invasive disease (Brueggemann et al. 2003).

1.7.3.2 Pathogenesis

S. pneumoniae normally resides on the mucosal surfaces in the upper respiratory tract where it can colonise asymptotically. However, if *S. pneumoniae* gains access to sterile tissue in the lower respiratory tract, then inflammatory responses can damage host tissues. These responses are characteristic of pneumonia. As the damaged tissue is more porous to microbes, these events can lead to further translocation of *S. pneumoniae* across epithelial barriers into the circulation causing bacteraemia. In turn, *S. pneumoniae* can translocate to the brain causing meningitis (Weiser, Ferreira, and Paton 2018). Transmission of *S. pneumoniae* occurs via respiratory droplets that exit from a host via a process called shedding (Kadioglu et al. 2008). Shedding is induced by inflammatory responses to *S. pneumoniae* or co-infections such as influenza virus (Weiser, Ferreira, and Paton 2018). Certain factors such as the pore-forming virulence factor pneumolysin (Ply) increase the shedding of bacteria which drives transmission (Zafar et al. 2017). Once transmitted, *S. pneumoniae* colonises the nasopharyngeal tract of a new host that can remain asymptomatic or lead to invasive disease.

To cause invasive disease *S. pneumoniae* must initially colonise the airway of the new host, invade host tissues and survive the attack from innate immune cells. Thus, epithelial, endothelial and immune cell barriers must be overcome to gain access to and survive in the bloodstream. To penetrate these barriers *S. pneumoniae* utilises both intracellular and paracellular invasion mechanisms by hijacking host recycling pathways and damaging epithelial layers. *S. pneumoniae* express a key virulence factor pneumococcal surface protein C (PspC) that binds polymeric immunoglobulin receptor (pIgR) expressed on epithelial cells initiating endocytosis (Zhang et al. 2000). Alternatively, *S. pneumoniae* can enter epithelial cells through expression of phosphorylcholine (ChoP) that binds host platelet-activating factor receptor (PAFR) (Cundell et al. 1995). Finally, the pneumococcal type-I pilus engages both pIgR and platelet endothelial cell adhesion molecule 1 (PECAM1) to cross the blood-brain barrier and cause meningitis, as demonstrated in mouse models (Iovino et al. 2017; 2020). Another strategy *S. pneumoniae* employ to invade tissue is through damaging and disrupting the integrity of epithelial layers by secreting cytotoxins and/or inducing inflammation. There are several virulence factors that damage epithelial layers facilitating paracellular invasion including cytotoxins such as Ply; H₂O₂-producing enzymes such as pneumococcal pyruvate oxidase (Spx) B and α -glycerophosphate oxidase (GlpO); metalloproteinases such as pneumococcal zinc metalloprotease (Zmp) A and ZmpB; proteinases such as endopeptidase (PepO) (Weiser, Ferreira, and Paton 2018).

PspC proteins – surface anchored proteins with immune evasion properties

PspC (also referred to as choline binding protein, CbpA; *S. pneumoniae* secretory IgA binding protein, (SpsA); factor H-binding inhibitor of complement (Hic); C3-binding protein A, PbcA) is a key virulence factor expressed on *S. pneumoniae* cell surfaces. PspC proteins are broadly expressed by *S. pneumoniae*, with most strains carrying at least one *pspC* gene (Iannelli et al. 2004). Loss of PspC expression abrogates *S. pneumoniae* virulence

with strains showing reduced colonisation of the nasopharyngeal tract and reduced recovery in the lungs and blood after intraperitoneal infection using a mouse model (Ogunniyi et al. 2007).

The main immune evasion mechanism proposed for PspC is through suppression of complement activation. PspC binds key components of the complement system such as FH, C3b, and C4BP (Andre et al. 2017). By sequestering FH and C4BP to its bacterial surface *S. pneumoniae* can inhibit C3b and C4b deposition. Reduced C3b and C4b inhibits activation of the alternative and classical pathway. Multiple studies have shown that PspC expression reduces C3b and C4b deposition after incubation of *S. pneumoniae* with blood plasma (Li et al. 2007; Peppoloni et al. 2006; Marshall et al. 2021; Yuste et al. 2010; Haleem et al. 2018).

In addition, PspC likely utilises a complement-independent immune evasion mechanism. A study using a complement-deficient mouse model revealed that *pspC* knockout *S. pneumoniae* strains exhibited attenuated virulence compared to wildtype (Kerr et al. 2006). Thus, PspC enhances *S. pneumoniae* virulence in mouse models independently of complement inhibition. PspC non-opsonically binds IgA, suggesting a potential mechanism to evade immune cell recognition (Dave et al. 2004). Furthermore, as previously discussed PspC binds polymeric immunoglobulin receptor (pIgR) expressed on epithelial cells initiating endocytosis, which may evade immune surveillance systems (Zhang et al. 2000).

Ply – a toxin that suppresses immune cells

Ply is a pore-forming toxin that is a key virulence factor expressed by all *S. pneumoniae* strains. Though generally localised to the bacterial cell wall, Ply is also secreted during bacterial growth (Price and Camilli 2009). Ply plays a remarkably diverse role in interacting with several host factors and distinct immune responses. Surface-expressed Ply directly binds host receptors expressed on immune cells to suppress inflammatory responses and evade immune responses. Notably, Ply binds MRC-1 expressed on human dendritic cells and murine alveolar macrophages where it suppresses the secretion of proinflammatory cytokines and TLR signalling (Subramanian et al. 2019). Ply binding to MRC-1 suppresses TLR4 signalling through upregulation of the cytokine suppressor SOCS1. Utilising a mouse model the data showed that Ply also promotes immune evasion through binding MRC-1 to promote internalisation into macrophages avoiding localisation to lysosomal compartments (Subramanian et al. 2019). Thus, through the Ply-MRC-1 interaction *S. pneumoniae* is able to evade immune responses.

Similar to other key immune evasion factors, Ply also interacts with key components of the complement system such as C1q, IgG, and L-Ficolin. However, Ply-mediated activation of complement appears to promote bacterial survival (Kuikka et al. 1992; Alcantara, Preheim, and Gentry-Nielsen 2001). Whilst this may appear counter-intuitive, multiple *in vivo* models have elucidated this immune evasion strategy. The Gentry-Nielsen group demonstrated that Ply induces complement activation which decreases C3 levels but also depletes total serum haemolytic content (Alcantara, Preheim, and Gentry-Nielsen 2001). Thus, whilst C3 is proteolytically cleaved and activated to produce C3b, the total levels of complement factors required to maintain robust responses are also

depleted. In summary, Ply is involved in multiple stages of pathogenesis including transmission, colonisation, and infection.

1.8 Hypothesis, aims, objectives

Given that human inhibitory receptors are targeted for direct interaction by human streptococcal pathogens and that inhibitory LILR are major regulators of innate immune cell responses, the hypothesis of the current investigation was that human Streptococcal pathogens target inhibitory LILR receptors for immune evasion purposes.

In order to assess this hypothesis the aims were:

- 1) Identify Streptococcal ligands that bind inhibitory Leukocyte Ig-like receptors (LILR)
- 2) Define the binding interface of LILR with identified ligands
- 3) Determine the functional consequences of ligand-LILR interaction for immune evasion
- 4) Explore additional immune evasion strategies utilised by Streptococcal pathogens

Chapter 2. *S. pyogenes* engages the inhibitory LILRB2 receptor via the M3 protein

2.1 Introduction

Streptococcus pyogenes is the causative agent in a range of diseases from relatively mild diseases such as pharyngitis and impetigo, to life-threatening invasive disease such as necrotizing fasciitis, bacteraemia, cellulitis, and streptococcal toxic shock syndrome (STTS) (Carapetis et al. 2005). Infections caused by *S. pyogenes* are a huge global health burden with 18.1 million severe cases recorded in 2005 resulting in at least 517, 000 deaths per year; of these, 160,000 deaths were due to invasive *S. pyogenes* diseases that have extremely high mortality rates of up to 45% (Craik et al. 2022; Brouwer et al. 2023; Carapetis et al. 2005). Despite this health burden, there is currently no vaccine available to protect against *S. pyogenes* infections and it is becoming more difficult to treat infections reflected by increasing reports of the emergence of antibiotic resistance in *S. pyogenes* (Brouwer et al. 2023). Thus, there is a crucial need to better understand disease pathogenesis to support the development of novel therapeutics. A key component of *S. pyogenes* pathogenesis is its ability to evade the immune system in order to survive, disseminate, and cause disease (Brouwer et al. 2023). Thus, investigating *S. pyogenes* immune evasion strategies can support the development of novel therapeutics.

S. pyogenes expresses surface-bound and secreted virulence factors that enhance immune evasion by inhibiting components of the immune system including immune cells, antibodies, complement factors, and antimicrobial peptides (Walker et al. 2014). Numerous virulence factors have been identified that promote immune evasion including *S. pyogenes* cell envelope protein (SpyCEP), hyaluronic capsule, streptolysin O (SLO), streptolysin S (SLS), DNase Sdal, Mac-1/2, and M protein (Walker et al. 2014; Smeesters, McMillan, and Sriprakash 2010; Metzgar and Zampolli 2011). It is likely that these immune evasion mechanisms work synergistically to suppress the innate immune response and thereby promote bacteria survival within the human host.

Neutrophils are by far the most abundant immune cells in circulation playing a key role in clearance of invasive pathogenic microbes. Thus, it is perhaps unsurprising that several *S. pyogenes* virulence factors involved in immune evasion directly or indirectly inhibit neutrophils (Fieber and Kovarik 2014). These *S. pyogenes* immune evasion factors typically disrupt important neutrophil functions, such as chemokine sensing, reducing opsonophagocytosis, suppressing degranulation, detoxifying reactive oxygen species (ROS), and inhibiting NET

degradation (Cole et al. 2010; Miyoshi-Akiyama et al. 2005; Kurupati et al. 2010; Döhrmann, Cole, and Nizet 2016). Arguably the most studied *S. pyogenes* virulence factor is the M protein. The *emm* gene that encodes M protein is highly polymorphic resulting in over 250 protein variants that each have different binding properties and host interaction (Smeesters, McMillan, and Sriprakash 2010). The M proteins are surface anchored proteins that have a coiled-coil structure and act as a major virulence factor of *S. pyogenes* by enhancing resistance to phagocytosis (Metzgar and Zampolli 2011). This is achieved by the propensity of M proteins to bind complement inhibitory factors such as C4BP, FH, and fibrinogen; and their propensity to non-opsonically bind IgG antibodies (Metzgar and Zampolli 2011). A second example of a well-characterised immune evasion factor is SpyCEP, a surface anchored protein that proteolytically cleaves and inactivates the chemokine interleukin (IL)-8 with the effect of inhibiting neutrophil activation and recruitment to sites of infection (McKenna et al. 2021; Edwards et al. 2005). Furthermore, *in vivo* studies using a soft tissue mouse model demonstrated that expression of SpyCEP was both necessary and sufficient for systemic bacterial dissemination (Kurupati et al. 2010). Thus, factors promoting *S. pyogenes* immune evasion of neutrophils play a central role in determining virulence and disease outcome.

LILRB2 is an important inhibitory immune receptor that modulates immune cell responses to bacteria. It is expressed by a range of myeloid cells involved in *S. pyogenes* immunity, including neutrophils, macrophages, monocytes and dendritic cells (Anderson et al. 2022). Importantly, LILRB2 agonism has been demonstrated to suppress anti-bacterial immune responses of innate immune cells including neutrophils, monocytes, macrophages, and dendritic cells (Abdallah et al. 2021). For example, in neutrophils the engagement of LILRB2 inhibits phagocytosis of *E. coli* and suppresses FcR-mediated activation and ROS production (Baudhuin et al. 2013). Likewise, on monocytes, LILRB2 agonism suppresses activation and phagocytosis (Fanger et al. 1998). Finally, on macrophages, LILRB2 antagonism promotes secretion of pro-inflammatory factors such as TNF- α (Chen et al. 2004). Thus, LILRB2 expressed on several myeloid cell surfaces plays an important role in modulating responses to bacteria, presenting a potential target for human pathogens including *S. pyogenes* to exploit for immune evasion.

As a potent inhibitory immune receptor expressed on innate immune cells, LILRB2 could be an attractive target for pathogens to suppress immune responses during infection. Indeed, LILRB2 is targeted by the human parasitic pathogen *Plasmodium falciparum* (Sakoguchi et al. 2021). *P. falciparum* is a protozoan that causes the most severe forms of malaria (falciparum malaria). During pathogenesis, *P. falciparum* invades erythrocytes inducing expression of a variety of RIFIN proteins on erythrocyte cell surfaces that promote immune evasion (Bachmann et al. 2015). Utilising a RIFIN expression library, the Saito group identified a specific RIFIN that interacts with LILRB2 (Sakoguchi et al. 2021). Although how the engagement of this RIFIN with LILRB2 impacts immune evasion is yet to be elucidated. Nonetheless, a previous study demonstrated that a different RIFIN from the expression library engages LILRB1 inhibiting activation of LILRB1-expressing NK cells and B-cells (Saito et al. 2017). As LILRB1 and LILRB2 have similar inhibitory signalling mechanisms and functional properties, it is expected that RIFIN-LILRB2 interactions will also inhibit activation of immune cells (Saito et al. 2017). Furthermore, a study of placental malaria revealed that monocytes from infants that developed active malaria expressed significantly

higher levels of LILRB2 compared with monocytes from individuals that that did not develop malaria (Dechavanne et al. 2022), supporting the proposition that *P. falciparum* may target LILRB2 for immune evasion.

Beyond being a direct molecular target of immune evasion strategies, it is emerging that LILRB2 plays a key role in determining outcome of infectious diseases. A study measuring LILRB2 expression in macrophages showed an upregulation of LILRB2 upon infection with *S. typhimurium* (Brown et al. 2009). However, the functional consequences of LILRB2 regulation for *S. typhimurium* immune evasion are yet to be determined. Furthermore, a study of 5,126 HIV-1 patients found that the strength of LILRB2 binding to HLA-B molecules was associated with increased viral replication of HIV-1 and disease progression (Bashirova et al. 2014). Thus, LILRB2 function and expression is associated with several infectious disease states suggesting pathogens potentially target LILRB2 for immune evasion.

A preliminary unpublished screen performed by the McCarthy group assessed pathogenic bacteria for interactions to a panel of recombinant LILR. Recombinant LILRB1-LILRB5 and LILRA1-LILRA6 were tested for binding to a range of pathogenic bacteria by flow cytometry. From this screen, LILRB2 was identified to have a potential interaction with *S. pyogenes*. Given that LILRB2 suppresses immune activation and function of several immune cells important for *S. pyogenes* immunity, the engagement of LILRB2 by *S. pyogenes* may represent a novel immune evasion mechanism. I sought to characterise and define the LILRB2-*S. pyogenes* interaction and determine the consequences for immune evasion. Identifying the *S. pyogenes* ligand(s) involved in binding LILRB2 may reveal novel *S. pyogenes* virulence factor(s) or provide further functional characterisation of existing *S. pyogenes* virulence factor(s). In addition, our investigations may provide the first evidence directly linking bacterial engagement of LILRB2 with immune evasion.

2.1.1 Hypothesis and aims

The hypothesis proposed was that *S. pyogenes* expresses a ligand(s) that engages LILRB2, and that these interactions promote immune evasion and bacterial survival during human infections. The aims were to (1) identify the *S. pyogenes* ligand that binds to LILRB2, (2) construct recombinant *S. pyogenes* ligand, and (3) explore the functional consequences of LILRB2 engagement for *S. pyogenes* in immune evasion.

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

E. coli strains (**Table 2.1**) were cultured in Lysogeny broth (LB, Invitrogen) or on LB agar plates (LB + 1.5% [w/v] bacterial agar VWR) at 37°C with shaking or statically, respectively. For strains containing pRSET and pOri23 vectors, LB was supplemented with 100 µg/mL ampicillin or 500 µg/mL erythromycin, respectively. *S. pyogenes* strains (**Table 2.1**) were cultured statically in Todd Hewitt (TH) (Millipore) supplemented with 1.5% yeast [w/v] (OXOID, THY) or on THY plates (THY + 1.5% [w/v] bacterial agar, VWR) at 37°C with 5% CO₂. The parental *L. lactis* MG1363 strain is previously described (Wegmann et al. 2007). *L. lactis* strains (**Table 2.1**) were cultured statically at 30°C in M17 broth supplemented with 0.5% [w/v] glucose and 0.5% [w/v] lactose (GM17) or on GM17 plates (GM17 + 1.5% [w/v] Bacterial Agar, VWR). Strains carrying pOri23 were cultured in GM17 supplemented with 5 µg/ml erythromycin.

Strain	Description	Reference
<i>Streptococcus pyogenes</i>		
5448	serotype; <i>emm1</i>	Sriskandan
950771	serotype; <i>emm3</i>	(Ashbaugh et al. 1998)
$\Delta emm3$ 950771	serotype; <i>emm3</i> with $\Delta emm3$ deletion	(Ashbaugh et al. 1998)
STAB-1118	serotype; <i>emm3</i>	Sriskandan
STAB-902	serotype; <i>emm3</i>	Sriskandan
H1569	serotype; <i>emm3</i>	Sriskandan
H387	serotype; <i>emm3</i>	Sriskandan
H517	serotype; <i>emm3</i>	Sriskandan
H518	serotype; <i>emm3</i>	Sriskandan
H672	serotype; <i>emm3</i>	Sriskandan
H673	serotype; <i>emm3</i>	Sriskandan
H987	serotype; <i>emm3</i>	Sriskandan
H1198	serotype; <i>emm3</i>	Sriskandan
H1200	serotype; <i>emm3</i>	Sriskandan
<i>Escherichia coli</i>		
For carriage of recombinant M protein vectors		
DH5a + pOri23. <i>emm3</i>	DH5a carrying pOri23.sM3 vector	This study
For expression of recombinant M proteins		
Rosetta Gami + pOri23. <i>emm3</i>	Rosetta Gami expressing rM3 His-tag protein	This study
<i>Lactococcus Lactis</i>		
For surface-expressed M proteins		
<i>L. lactis</i> + pOri23. <i>emm1</i>	<i>L. lactis</i> MG1363 carrying pOri23. <i>emm1</i> vector	Sriskandan
<i>L. lactis</i> + pOri23. <i>emm3</i>	<i>L. lactis</i> MG1363 carrying pOri23. <i>emm3</i> vector	Sriskandan

Table 2.1. Bacterial strains used in Chapter 2.
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2.2.3 Construction of expression vectors

Preparing chemically competent E. coli

Overnight cultures of DH5 α , TOP10, and RG strains were inoculated 1:100 into 200 ml SOB [(Bacto Tryptone (Peptone) 5 g, yeast 1.25 g, 5 M NaCl 0.5 ml (146 mg), 1 M KCl 0.625 ml (46 mg), 1 M MgCl₂ 2.5 ml (508 mg), 1 M MgSO₄ 2.5 ml (610 mg)] and grown at 37°C until they reached an absorbance at 600nm wavelength (OD₆₀₀) of 0.5. Cultures were then incubated on ice for 30 mins, centrifuged at 1455 *g* for 15 mins at 4°C, resuspended in 66 ml ice cold RF1 (RbCl 2.4 g, MnCl₂·4H₂O 1.98 g, CH₃CO₂K 0.589 g, CaCl₂·2H₂O 0.3 g, glycerol 30 g, brought to pH 5.8 with 0.2 M acetic acid) and incubated on ice for 60 mins. The bacteria were then pelleted by centrifugation at 1455 *g* for 15 mins at 4°C resuspended in 16 ml of ice cold RF2 (MOPS 0.209 g, RbCl 0.12 g, CaCl₂·2H₂O 1.10 g, glycerol 15 g, brought to pH6.8 using 1 M NaOH), incubated on ice for 15 mins, aliquoted, snap frozen with liquid nitrogen, and stored at -80°C.

Transforming E. coli strains for expression of pOri23.emm3-His vector

DH5- α or TOP10 *E. coli* strains (**Table 2.1**) were transformed to overexpress pOri23.emm3-His (**Table 2.2**), by heat shock. Chemically competent *E. coli* (50 μ L) was incubated with 50-100 ng pOri23.emm3-His for 20 mins on ice, 1 min 42°C, 2 mins on ice, and finally 120 mins at 37 °C. Cultures were spread on LB + 500 μ g/ml erythromycin agar plates and incubated at 37°C overnight for selection of pOri23 transformants. Transformants containing pOri23.emm3-His vectors were screened by colony PCR using DreamTaq Green PCR Master Mix 2 $\times$ (Thermo Fisher Scientific). For the reaction mixture, 1 μ l of forward primer (20 mM) (**Table 2.3**), 1 μ l of reverse primer (20 mM), 1 μ l of lysed bacterial colony previously resuspended in 20 μ l milliQ, 12.5 μ l DreamTaq Green polymerase (Thermo Fisher Scientific), and 9.5 μ l dH₂O water were mixed. PCR was set to 95°C for 5 mins, 35 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 60 s, 72°C for 10 mins. PCR products were separated on a 1.25% agarose gel by gel electrophoresis to assess the size of the vector insert.

Vector	Description	Reference
<i>For expression of recombinant M3-His protein in L. lactis</i>		
pOri23- <i>emm3</i> -His	pOri23 carrying extracellular domain of <i>emm3</i> gene under control of p23 promoter	This study

Table 2.2. Vectors used in Chapter 2.

Oligonucleotide	Sequence (5' - 3'; alanine substitution underlined)
<i>For construction of recombinant M3-His protein</i>	
pOri23- <i>emm3</i> -His-F	TATGAATGACAATGATGTTGGATCCATGGCTAAAAACAACACGAA
pOri23- <i>emm3</i> -His-R	ATCGATAAGCTTGGCTGCAGGTCGACTTAGTTTCTTCTTGCGTT
<i>To confirm insertions into pOri23 vector</i>	
pOri23-F	TCAGACCTAAGACTGATGACAAAAAGAG
pOri23-R	TGTGGATAACCGTATTACCGCCTTTGAGTG

Table 2.3. Primers used in Chapter 2.

Construction of recombinant M3 His-tag protein vector

A pOri23 *E. coli*-*L. lactis* shuttle vector was used that enable expression of proteins under the p23 promoter at high levels in *L. lactis*. To produce a vector expressing recombinant M3-His, it was first necessary to construct a template vector containing full length *emm3* gene with a C-terminal His-tag. This was kindly performed by the Srisikandan lab. In brief, PCR was used to amplify the *emm3* gene from STAB-902 strain and Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). The template DNA was prepared by boiling one colony of the *S. pyogenes* strain in 50µl of dH2O for 5 minutes. The reaction conditions were 12.5 µl Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), 9.5 µl of milliQ, 1 µl forward primer at 20 mM, 1 µl reverse primer (20mM), and 1 µl of *S. pyogenes* DNA. Standard protocol reaction mixtures were used with thermocycling conditions of 2 mins at 98°C, 35 cycles of 15 s at 98°C, 30 s at 62°C, and 1 min at 72°C, followed by 5 mins at 72°C. Amplicons were separated on a (1% w/v) agarose gel. DNA of an amplicon of expected size (1.6kb) was purified from the 1% (w/v) agarose gel using GeneJET Gel Extraction Kit (Thermo Fisher Scientific), digested with

BamHI and SalI (New England Biolabs), and ligated into BamHI/SalI linearized pOri23 vector following manufacturer's protocols. The ligation reaction was subsequently transformed into *E. coli* DH5 α cells, and transformants were selected for by growth at 37°C on Luria-Bertani (LB) agar supplemented with 500 μ g/ml erythromycin. Transformants containing pOri23 vectors with the full length *emm3* gene (pOri23.*emm3*-wildtype) were screened by colony PCR using DreamTaq Green PCR Master Mix 2 $\times$ (Thermo Fisher Scientific) and primers amplifying across the insertion site of pOri23 (**Table 2.3**). To produce pOri23.*emm3*-His vector, PCR was used to amplify the extracellular region of the *emm3* gene using primers (**Table 2.3**) and Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). The same method using the same concentrations and conditions were used as described above.

Producing chemically competent L. lactis and transformation

Competent *L. lactis* were produced by subculturing *L. lactis* in GM17 media supplemented with 0.5 M sucrose at 30°C until mid-logarithmic phase (absorbance at OD₆₀₀ = 0.7). *L. lactis* was then incubated at 4°C for 10 mins, centrifuged at 3273 *g* for 15 min, washed three times by resuspending in 3 ml Electroporation Buffer (0.5 M sucrose and 10% [v/v] glycerol), centrifuged at 3,273 *g* for 15 mins, and resuspended in 100 μ L electroporation buffer.

100 μ L of competent *L. lactis* was transformed with 5 μ L pOri23 vectors by electroporation using a 1-mm gap electroporation cuvette at 1.2 kV and recovered by growth in 900 μ L M17 medium supplemented with 0.5 M sucrose, 0.5% (w/v) glucose, 20 mM MgCl₂, and 2 mM CaCl₂ on ice for 10 mins followed by incubation at 30°C for 2 h. Transformants were selected by growth at 30°C on GM17 agar plates supplemented with 5 μ g/ml erythromycin after 48 h.

Total M3 protein expression by bacteria was measured by SDS-PAGE analysis of cellular lysates. 1 ml of overnight bacterial culture was pelleted by centrifugation (5 mins, 3,273 *g*), then resuspended in 100 μ L sample buffer, and lysed by heating to 95°C for 10 mins. Samples were separated on a 12.5% SDS-PAGE gel, washed in double-distilled H₂O, stained with Biosafe Coomassie Blue stain (Bio-Rad Laboratories), and destained with double-distilled H₂O.

2.2.4 Expression and purification of recombinant M3 protein

L. lactis was used to overexpress recombinant M3 with a C-terminal His tag (rM3-His) using a pOri23 shuttle vector (Que et al. 2000). Overnight cultures of *L. lactis* carrying pOri23.*emm3*-His vector (**Table 2.1**) were subcultured 1:20 in 300 ml of GM17 + 5 μ g/ml of erythromycin and incubated aerobically at 30°C overnight. To

assess whether rM3-His was retained in the *L. lactis* cytoplasm or secreted into the supernatant, 100 µl samples of bacterial culture were centrifuged at 3273 *g* for 5 mins and the pellet and supernatant separated. Protein expression was measured by adding 100 µl of sample buffer to each sample, then boiling at 95°C for 5 mins, before running samples on an 12.5% SDS-PAGE gel for 45 mins at 270 V. The SDS-PAGE gel was washed in dH₂O 3 times for 5 mins, stained with Coomassie blue stain (ThermoFisher) for 30 mins, and finally washed 3 times in dH₂O before imaging. Assessment of the location of rM3-His revealed the target protein was secreted into the supernatant. Thus, the supernatant of the 300 ml cultures was collected by centrifuging the cultures at 3,273 *g* for 30 mins at 4°C and samples were store at 4°C until purification.

rM3-His was concentrated in the supernatant by centrifugal filtration. For this procedure, the supernatant of the overnight cultures was subjected to centrifugal filtration using 10 kDa centrifugal filters (Amicon) to remove secreted molecules of <10 kDa from the supernatant. Centrifugal filters were initially washed, by adding 15 ml of dH₂O water and centrifuging for 10 mins at 3,273 *g*. To purify and concentrate the supernatant, each centrifugal filter tube was supplemented with 15 ml of PBS and then centrifuged for 30 mins at 3,273 *g*. The solution that was retained by the centrifugal filter was collected and further supplemented with 15 ml of PBS. This purification process was repeated 10 times for each 15 ml sample.

rM3-His was purified from the concentrated supernatant samples by affinity chromatography (ÄKTA pure; GE Healthcare Life Sciences) using 5 ml His-Trap Nickel columns (Cytiva, Life Sciences). Prior to use, Nickel columns were washed by passing 25 ml of native binding buffer (20 mM Na₂HPO₄ 0.5 M NaCl, pH 7.8) over the His-Trap column using a peristaltic pump. To load the His-Trap columns with rM3-His, supernatant samples were passed over His-Trap Nickel column, followed by 25 ml of native binding buffer, (20mM Na₂HPO₄, 0.5 M NaCl, pH 7.8) using a peristaltic pump. rM3-His was eluted from the His-Trap Nickel column by passing a gradient of imidazole (10-500 mM) over the His-Trap column and collecting 1.5 ml elution fractions. As the ÄKTA pure collects and plots data on absorbance of elutions over time (UNICORN system control software), the absorbance curve was used to predict the elution fractions that contained rM3-His. For fractions selected based on absorbance (50 µl of the elution sample was mixed with 50 µl of 2 x sample buffer, boiled at 95°C for 5 mins, and tested for protein expression by SDS-PAGE analysis as previously described. Elution samples containing a detectable concentration of protein on the stained SDS-PAGE gel at ~60 kDa (the predicted molecular weight of rM3-His) were pooled and dialysed in 1X PBS at 4°C overnight in 12 kDa dialysing tubes (SLS). Protein samples were concentrated by centrifugation at 3,273 *g* using 10 kDa filter concentrating units (Amicon) to volumes of 1-5 ml and samples were stored at -20°C.

2.2.5 Biochemical assays

Binding of rLILRB2-Fc to rM3-His protein coated Dynabeads

The interaction of rLILRB2-Fc with Dynabeads coated with rM3-His protein was measured by flow cytometry. To prepare rM3-His protein coated Dynabeads, His-Tag Dynabeads (Invitrogen; 0.4 µL per sample) were washed once in wash buffer (1X PBS containing 0.5% (w/v) BSA (Sigma Aldrich) + 10 mM imidazole (Sigma Aldrich)). Dynabead washes were performed by addition of 100 µl wash buffer, transference to a magnet for 2 mins, aspiration of wash buffer, and resuspension in 100 µl wash buffer. Dynabeads were coated in rM3-His protein by incubation with 160 ng of rM3-His (diluted to 100 µg/ml in 1X PBS + 0.5% BSA + 10 mM imidazole) per sample for 30 mins on ice. Dynabeads were then washed twice in wash buffer to remove unbound rM3 His-tag protein. To measure the rLILRB2-Fc interaction with rM3-His coated Dynabeads, 30 µL of a 10 µg/mL solution of rLILRB2-Fc, rLILRB3-Fc, or rCEACAM1-Fc diluted in wash buffer was incubated with the rM3-His coated Dynabeads and incubated on ice for 1 hour at 37°C. Subsequently, the Dynabeads were washed 3 times in wash buffer and stained with goat anti-human-IgG-FITC (1:1,000; Southern Biotech) for 30 mins on ice. Dynabeads were then washed 3 times in wash buffer and resuspended in 30 µl of the same buffer. Fluorescence was measured by flow cytometry FACS Calibur (Becton Dickinson) and analysed by FlowJo.

2.2.6 Bacterial cell culture assays

*Measuring M3 expression of *L. lactis* by Western blot*

L. lactis (pOri23.*emm1* and pOri23.*emm3*) and *S. pyogenes* (950771) strains were probed for M3 expression using mouse anti-M3 serum that was supplied by Gunnar Lindahl (Lund University, Sweden). Overnight stationary phase *L. lactis* or *S. pyogenes* cultures were adjusted to OD₆₀₀ =1.2 and 300 µl were pelleted by centrifugation for 5 mins at 3,273 g, and resuspended in 25 µl of 1X Sample buffer (0.25M Tris (pH 6.8), 10% SDS, 12% glycerol, 0.5% bromophenol blue; 5% β-mercaptoethanol). The samples were then boiled for 10 mins at 95°C and proteins were separated by running on a 12.5% SDS-PAGE at 270 V for 45 mins. The proteins were transferred from the 12.5% SDS-PAGE gel to 0.45 µM nitrocellulose membrane in a TransBlot Turbo (BioRad) for 40 mins at 60 V. After transfer, the membrane was briefly rinsed in TBS-T (1X Tris-buffered Saline (Sigma) + 0.1% Tween (Sigma)) before being blocked for 2 hours at room temperature in 10 ml blocking buffer (5% milk in TBS-T). The nitrocellulose membrane was washed 5 times (for 5 mins in 5 ml TBS-T) and then stained with 5 ml mouse anti-M3 serum (diluted 1:3,000 in blocking buffer) overnight at 4°C. The next day, the nitrocellulose membrane was washed 5 times (for 5 mins in 5 ml TBS-T) and incubated with goat anti-mouse-HRP secondary antibody (diluted 1:10,000 in blocking buffer) at room temperature for 1 hour. Finally, nitrocellulose membrane was washed 5 times in TBS-T. Bands were visualised by using a ChemiDoc XRX+ (BioRad) following a one-minute incubation of the membrane with ECL Western Blotting Substrate (Promega).

Measuring L. lactis M3 surface expression by flow cytometry

The surface expression of M3 protein on *L. lactis* and *S. pyogenes* strains was measured by flow cytometry. Overnight bacterial cultures were pelleted at 3,273 g for 10 mins at 4°C and resuspended in 1X PBS + 0.5% BSA to an OD₆₀₀ of 0.4. Bacterial suspensions (15 µl) were added to wells of a 96-well U-bottom plate and incubated with an equal volume of 0.05% (w/v) mouse anti-M3 sera or normal mouse sera for 1 hour on ice. Following incubation, bacteria were washed once in ice cold PBS plus 0.5% (w/v) BSA and pelleted at 2,250 g for 5 mins at 4°C. Bacteria were resuspended in 20 µl goat anti-mouse-IgG-FITC (Life Technologies) diluted 1:1,000 in 1X PBS + 0.5% BSA for 45 mins on ice. Bacteria were washed (100 µl of PBS + 0.5% BSA at 2,250 g for 5 mins) and resuspended in 100 µl of the same buffer. Fluorescence of bacteria were measured by flow cytometry on a FACS Calibur and analysed by FlowJo.

Measuring LILRB2-Fc/LILRB2-His interaction with S. pyogenes and L. lactis by flow cytometry

The LILRB2 binding phenotype of *S. pyogenes* and *L. lactis* strains was measured by flow cytometry. Overnight cultures of stationary phase *S. pyogenes* and *L. lactis* were resuspended in 1X PBS + 0.5% BSA to an absorbance at OD₆₀₀ of 0.4. To measure rLILRB2-Fc binding, 30 µL of bacterial cultures were incubated with either 10 µg/ml rLILRB2-Fc, rLILRB1-Fc, or PBS for 30 mins on ice, washed (100 µl of PBS at 2,250 g for 5 mins), and stained with 30 µl 1:1,000 goat anti-human-IgG-FITC (Life Technologies). To prepare samples for FACS, bacteria were washed and fixed in 1% (v/v) PFA and stored at 4°C. Fluorescence of bacteria was measured by flow cytometry analysis (FACS Calibur). To quantify rLILRB2-His binding to *S. pyogenes* and *L. lactis* strains, a similar approach was used. Here, 30 µL of diluted and washed bacterial cultures were incubated with either 10 µg/ml rLILRB2-His, rLILRB1-His or PBS for 30 mins on ice, washed in PBS (100 µl of PBS at 2,250 g for 5 mins), stained with 1:50 anti-His-FITC (ThermoFisher), then fixed in 1% (v/v) PFA. Fluorescence of bacteria was measured by flow cytometry analysis (FACS Calibur). For rLILRB2-His and rLILRB2-Fc titration curves, the same approaches were followed but bacteria were resuspended in 30, 10, 3, 1, 0.3, 0.1, 0.03, and 0.01 µg/ml of rLILRB2-His, or rLILRB2-Fc.

Measuring LILRB2-His interaction with trypsin-treated S. pyogenes

The effect of trypsin treatment for abrogating LILRB2 binding phenotype of *S. pyogenes* and *L. lactis* strains was measured by flow cytometry. Overnight stationary phase *S. pyogenes* and *L. lactis* cultures at OD₆₀₀ of 0.4 were resuspended in 50 µl PBS + 0.5% trypsin EDTA (10x) (Sigma) and incubated for 30 mins at 37°C. Subsequently, bacteria were washed 5 times with PBS (100 µl of PBS at 2,250 g for 5 mins). Next, the binding of 10 µg/ml of rLILRB2-His and rLILRB1-His to the bacterial surface was measured using the method described above (under, “Measuring LILRB2-Fc/LILRB2-His interaction with *S. pyogenes* and *L. lactis* by flow cytometry”).

Measuring L. lactis and S. pyogenes collagen adhesion

Bacterial adhesion to collagen coated plates was measured by microplate reader (Bio-Rad). Wells of a 96-well, flat bottom plate were coated in 50 µl of 10 µg/ml of collagen I (Sigma), collagen IV (Sigma), or BSA (Sigma) and incubated at 4°C overnight. Wells were washed with 100 µl PBS, and subsequently blocked with 50 µl of PBS + 0.5% BSA for 1 hour at room temperature. Overnight *S. pyogenes* and *L. lactis* were sub-cultured 1:10 in THY or GM17 + 5 µg/ml erythromycin, respectively, and cultured until they reached an OD₆₀₀ = 0.4. 50 µl of mid-log phase bacteria at 3×10^8 CFU/ml, were added to each well, pelleted at 563 g for 4 mins, and incubated at 37 °C (*S. pyogenes*) or 30 °C (*L. lactis*) for 1 hour. To measure adherent bacteria, wells were washed 4 times by addition of 100 µl PBS then inversion of plate. After the washes, plates were stained with 50 µl of 0.5% crystal violet (Sigma) and incubated for 1 min at room temperature. Finally, wells were washed again 4 times (100 µl PBS and inversion). Fluorescence was measured by microplate reader at an absorbance of 595nm (Bio-rad). For rLILRB2-His inhibition of *L. lactis* adhesion, bacterial samples were pre-incubated with 10 µg/ml of rLILRB2-His, rLILRB1-His or PBS, before adding to collagen- or BSA-coated plates.

2.2.7 Ex vivo human blood assays

Ethical approval and consent for use of human blood

Ethical approval for the isolation and use of human blood was obtained from the Regional Ethics Committee and Imperial College Healthcare NHS Trust Tissue Bank (Regional Ethics Committee approval no. 17/WA/0161, Imperial College Healthcare Tissue Bank Human Tissue Authority license no. 12275, and Imperial College Research Ethics Committee no. 19IC5166).

Whole blood growth assays with S. pyogenes

Growth of *S. pyogenes* strains was assessed in whole human blood based on previous assays (Turner et al. 2015; 2020). The inoculum concentration was determined by assays (not shown) optimising for *S. pyogenes* growth in whole blood after 3 hours. Overnight *S. pyogenes* cultures were subcultured 1:10 in THY and grown to an OD₆₀₀ = 0.4. 270 µl whole blood and 15 µl bacteria (mid-logarithmic phase bacteria resuspended to 1×10^7 CFU/ml in HBS⁺⁺ (HEPES buffered saline) buffer) were mixed in a sterile 1.5 ml Eppendorf tube and incubated at 37°C with rotation for 3 hours. Next, samples were serially diluted in sterile PBS, plated onto THY agar plates, and incubated overnight at 37°C before counting CFUs. To measure rLILRB2-His blocking effects, *S. pyogenes* inocula were pre-incubated in 50 µl of 30 µg/ml rLILRB2-His, rLILRB1-His or PBS for 30 mins on ice. 30 µg/ml was used to ensure complete blocking and saturation of LILR-binding ligands expressed on *S. pyogenes* surfaces based on previous LILR binding titration assays. Bacteria were washed (100 µl PBS at 2,250 g for 5 mins) and resuspended to 2×10^7 CFU/ml in HBS⁺⁺ buffer.

Whole blood killing assays with *L. lactis*

Survival of *L. lactis* strains were assessed in whole human blood. The inoculum concentration was determined by assays (not shown) optimising for measurable differences in *L. lactis* killing between strains and/or conditions in whole blood after 30 mins, 60 mins, 90 mins, and 120 mins incubation. Overnight *L. lactis* were subcultured 1:10 and grown to an absorbance at OD₆₀₀ of 0.4. 150 µl whole blood and 50 µl bacteria (mid-logarithmic phase bacteria resuspended to 1×10^7 CFU/ml in HBS⁺⁺ buffer) were mixed in a 1.5 ml Eppendorf tube and incubated at 37°C with rotation for 120 mins incubation. Samples were taken every 30 mins until a final timepoint of 120 mins. Samples were serially diluted in sterile PBS, plated on GM17 agar plates, incubated overnight at 30°C, and CFUs counted. To measure rLILRB2-His blocking effects, *L. lactis* inocula were pre-incubated in 50 µl of 30 µg/ml rLILRB2-His, rLILRB1-His or PBS for 30 mins on ice. Bacteria were washed (100 µl PBS at 2,250 g for 5 mins) and resuspended to 2×10^7 CFU/ml in HBS⁺⁺ buffer.

2.3 Results

2.3.1 LILRB2 interacts with *emm3 S. pyogenes* strains

S. pyogenes strains are classified into >250 serotypes and genotypes based on expression of the M protein encoded by the *emm* gene (Brouwer et al. 2023). However, despite the huge diversity of M proteins, the vast majority of invasive infections are caused by *emm1*, *emm3*, *emm12*, *emm28*, and *emm89* strains (Koutouzi et al. 2015). Indeed, *emm1* and *emm3* strains are estimated to account for a third of clinical isolates in high income countries and are associated with outbreaks of *S. pyogenes* disease (Happonen et al. 2019). The dominance of *emm1* and *emm3* strains during outbreaks in scarlet fever and invasive disease is likely due to acquisition of immune evasion phenotypes and/or virulence traits. The hypothesis postulated was that *S. pyogenes* strains of *emm1* and *emm3* lineages interact with LILRB2. The McCarthy lab had previously produced purified a panel of rLILR-His proteins and identified a putative interaction of rLILRB2-His, but not rLILRB1-His, with *S. pyogenes*.

To test this hypothesis, the binding of rLILRB2-His to representative *S. pyogenes* strains from these lineages was measured. This included the well characterised M1 strain 5448 (necrotizing fasciitis and streptococcal toxic shock syndrome, (Chatellier et al. 2000)) and M3 strain STAB-902 (superficial pyoderma, (Soriano et al. 2014)). These strains were incubated with rLILRB2-His, rLILRB1-His, or PBS, and the deposition of His-tagged proteins on the bacterial surface was measured using anti-His-FITC secondary mAb and flow cytometry analysis (**Fig 2.1A-B**). Purified rLILRB-His was further tested for purity by SDS-PAGE (**Supp.2.1**). rLILRB2-His bound to the *emm3* strains – STAB-902, STAB-1118, and H1569; but not to the *emm1* strain 5448. rLILRB1-His did not bind to *emm1* or

emm3 *S. pyogenes* strains as expected and acted as a protein control (**Fig 2.1A-B**). Because rLILRB2-His bound to all three *emm3* *S. pyogenes* strains, the data suggest that rLILRB2-His binds to a putative ligand(s) expressed specifically by *S. pyogenes* strains belonging to the *emm3* serotype.

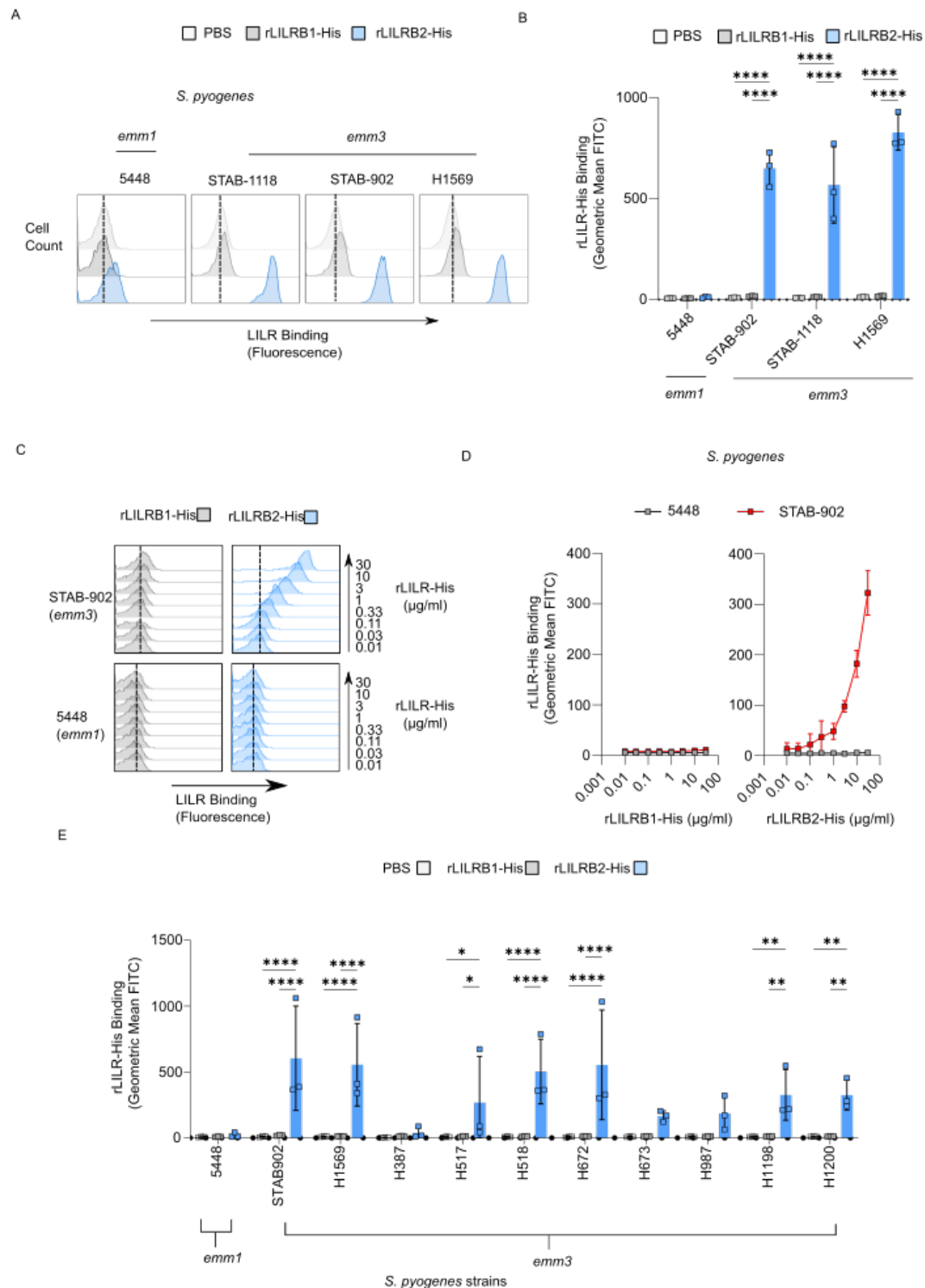


Figure 2.1. *S. pyogenes* carrying *emm3* gene bind LILRB2. (A-B) Binding of recombinant LILRB2 (rLILRB2-His) to the surface of *S. pyogenes* strains was measured by flow cytometry. Stationary phase *S. pyogenes* strains 5448, STAB-902, STAB-1118, and H1569 were incubated with rLILRB2-His (10 µg/ml), rLILRB1-His, or PBS for 30mins, and rLILRB1-His and rLILRB2-His binding was detected using anti-His-FITC (1:50). Representative flow cytometry plot (A) and summary graph (B) of 3 biological replicates. (C-D) Dose-dependent binding of rLILRB2-His to STAB-902 (*emm3*) and 5448 (*emm1*) *S. pyogenes* strains was assessed by performing a titration curve. Representative flow cytometry plot (C) and summary graph (D) shown represent 3 biological replicates. (E) A panel of *S. pyogenes* strains carrying the *emm3* gene were assessed for LILRB2 binding. Bacteria were incubated with rLILRB2-His (10 µg/ml), or rLILRB1-His (10 µg/ml), or PBS for 30mins, and rLILRB2-His binding was detected using anti-His FITC (1:50). In (B) and (E) graphs represent the mean ± standard deviation, and a two-way ANOVA was performed to assess statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Next, the specificity of rLILRB2-His binding to the *emm3* strain STAB-902 was tested. Here, STAB-902 and 5448 were incubated with increasing concentrations of rLILRB2-His. Binding of LILRB2-His to the bacterial surface was measured by flow cytometry (Fig 2.1C-D). The titration curve generated shows that rLILRB2-His, but not rLILRB1-His binds to STAB-902 in a dose-dependent manner (Fig 2.1D). In contrast, neither protein bound to strain the M1 strain 5448 within the concentration range tested. This demonstrates the binding of rLILRB2-His to the STAB-902 strain is specific.

Finally, rLILRB2 binding to all strains within the M3 serotype was assessed. rLILRB2-His and rLILRB1-His binding to a wider repertoire of *emm3* *S. pyogenes* strains (Fig 2.1E), kindly provided by the Srisikandan group at Imperial College London, was tested. rLILRB2-His binding to *emm3* strains STAB-902, H1569, H517, H518, H672, H1198, and H1200 was statistically significant compared to the rLILRB1-His control. rLILRB2-His also bound to M3 strains H673 and H987. However, the difference was not statistically significant. In contrast, rLILRB2-His did not bind to the M1 H387 strain. Differences in rLILRB2-His binding between *emm3* strains may be explained by variations in the expression levels of a putative LILRB2 ligand(s). Alternatively, *emm3* strains tested may have phenotypic differences that influence rLILRB2-His interaction. For example, *S. pyogenes* strains could express varying amounts of capsule that encase the bacteria forming a protective layer; and high levels of capsule may block rLILRB2-His interaction with the putative ligand. In conclusion, rLILRB2-His binds to multiple *emm3* strains to varying degrees, which may be explained by differences in expression levels of a putative LILRB2 ligand(s) and/or phenotypic differences that reduce access to a putative LILRB2 ligand(s).

2.3.2 *S. pyogenes* bind LILRB2 through a trypsin sensitive surface protein

The interaction of LILRB2 with the surface of M3 serotype *S. pyogenes* strains could be mediated by surface proteins, capsule (composed of hyaluronic acid), or the cell wall (composed of teichoic acid). As streptococcal

pathogens are known to interact with inhibitory immune receptors through proteins (Avondt, Sorge, and Meyaard 2015), the hypothesis that a surface protein formed the interaction with LILRB2 was tested by determining whether binding of rLILRB2-His to the *S. pyogenes* surface was impacted by pre-incubation of bacteria in trypsin that removes surface bound proteins. Trypsin enables cleavage of *S. pyogenes* surface proteins, including M protein, whilst not affecting cell viability (Severin et al. 2007), by cleaving peptide bonds at the C-terminal site of lysine and arginine residues, unless the preceding amino acid is proline (Manea et al. 2007). STAB-902 (*emm3*) and 5448 (*emm1*) were treated with either PBS or trypsin and then incubated with rLILRB2-His or rLILRB1-His. Protein binding to the bacterial surface was measured by flow cytometry (**Fig 2.2A-B**). The binding of rLILRB2-His to the surface of M3 STAB-902 was significantly reduced following pre-treatment of the *S. pyogenes* culture with trypsin (**Fig 2.2B**, bottom right panel). In contrast, there was no binding of rLILRB2-His to PBS treated M1 5448 and no binding of rLILRB1-His to either strain (**Fig 2.2B**). Thus, these data show that trypsin treatment of *S. pyogenes* completely abrogates binding of rLILRB2-His, suggesting that LILRB2 binds *S. pyogenes* via one or more surface expressed trypsin-sensitive protein(s).

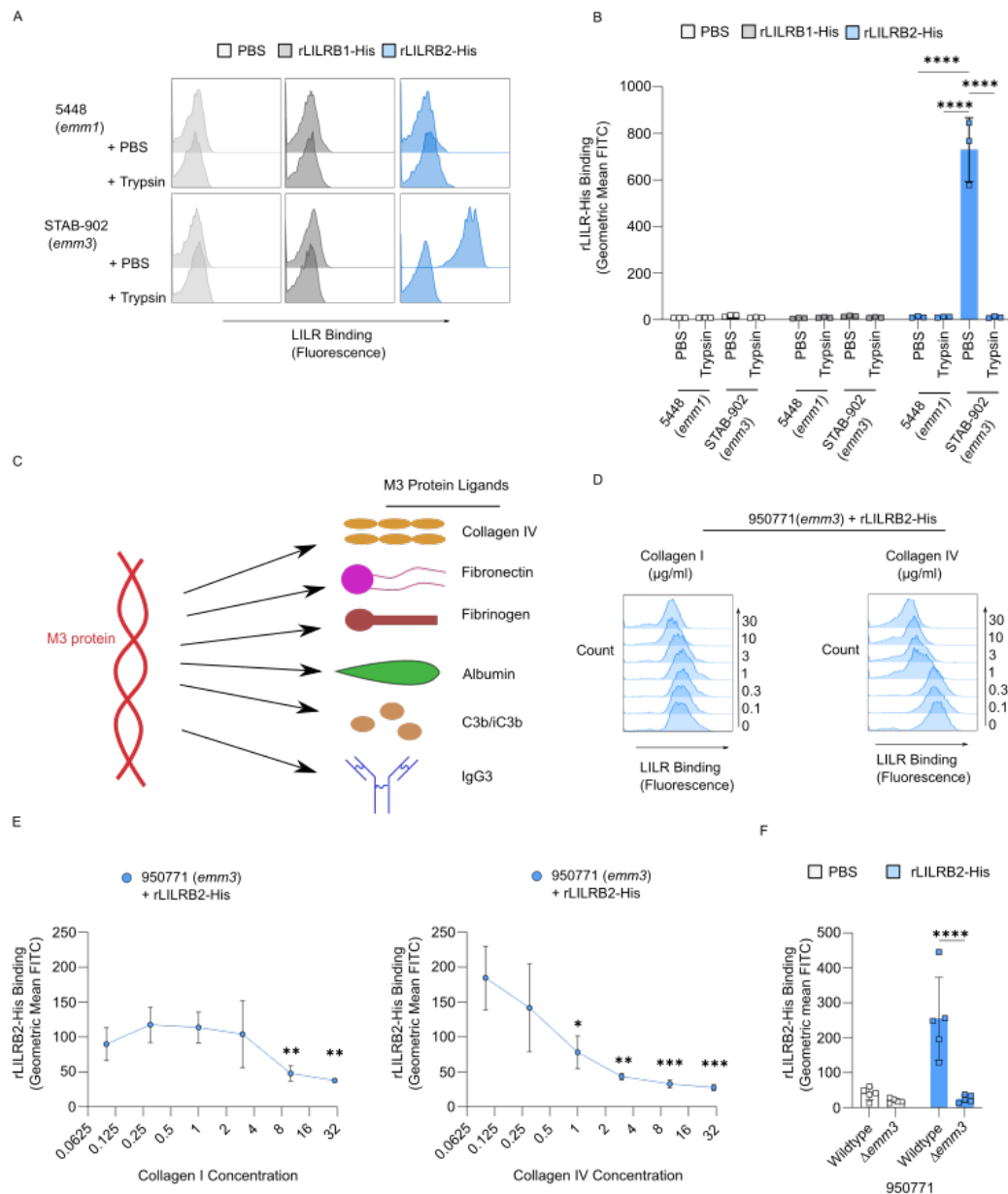


Figure 2.2. LILRB2.His interacts with *S. pyogenes* M3-serotype surface protein(s), that are blocked by collagen. (A-B) Stationary phase *S. pyogenes* strains were treated with trypsin (0.5%) or PBS, and rLILRB2-His surface binding was measured. Representative flow cytometry plot (A) and summary graph (B) of 3 biological replicates. (C) Schematic of M3 protein and the known M3 ligands. (D-E) Stationary phase 950771 (*emm3*) strain was incubated with increasing concentrations of collagen I and IV and rLILRB2-His surface binding was measured by flow cytometry. Representative flow cytometry plot (D) and summary graph (E) of 3 biological replicates. (F) 950771 isogenic strains were tested for rLILRB2-His binding. Stationary phase 950771 carrying *emm3* (wildtype) or *emm3* knockout (Δemm3) were incubated with rLILRB2-His (10 $\mu\text{g/ml}$) or PBS for 30 mins, and rLILRB2-His binding was detected using anti-His-FITC (1:50) by flow cytometry. Mean $\pm$ standard deviation was plotted, and two-way ANOVA (B and F) or one-way ANOVA (E) was performed to assess statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$).

The major surface protein of *emm3 S. pyogenes* is the M3 protein. The M3 protein is a surface protein that is a key virulence factor involved in adhesion and invasion of epithelial cells (Eyal et al. 2003). Unlike other M proteins, M3 has not yet been described to possess anti-phagocytic properties to date (Carlin, Chang, et al. 2009; Smeesters, McMillan, and Sriprakash 2010; Sandin, Carlsson, and Lindahl 2006). M3 protein is expressed as a homodimer and is a highly diverse molecule that binds to several ligands including collagen IV, fibronectin, fibrinogen, albumin, complement factors, and IgG antibodies (Smeesters, McMillan, and Sriprakash 2010) (**Fig 2.2C**). Because rLILRB2-His bound to all M3 serotype strains via a surface protein, M3 is likely the LILRB2 ligand expressed on the surface of *emm3 S. pyogenes*. To test whether the M3 protein was indeed the target of LILRB2, the competitive inhibition of rLILRB2-His to *S. pyogenes* using a characterised M3 ligand was assessed. Collagen IV was chosen as an M3 ligand, because M3 protein has been shown to directly interact with both soluble and bound collagen IV (Dinkla et al. 2003). In addition, collagen I was chosen as it has a similar amino acid composition to collagen IV and also binds to *emm3 S. pyogenes* strains (unpublished data, Sriskandan group) (Nguyen et al. 2012).

To test whether collagen I and IV acted as competitive inhibitors of rLILRB2-His binding to the surface of *emm3 S. pyogenes*, 950771 was pre-incubated with increasing concentrations of collagen I or collagen IV and measured rLILRB2-His binding to the bacterial surface by flow cytometry (**Fig 2.2D-E**). 950771 strain was chosen in favour of STAB-902 because an isogenic *emm3* knockout mutant was available. Incubation of strain 950771 with 10 µg/ml and 30 µg/ml of collagen I significantly inhibited rLILRB2-His binding to the bacterial surface (**Fig 2.2E**). Of note, collagen IV was a more effective inhibitor of rLILRB2-His binding than collagen I, with a lower minimum threshold (1 µg/ml) required to significantly inhibit rLILRB2-His binding to 950771 (**Fig 2.2E**). These data demonstrated that both collagen I and collagen IV effectively block the binding of rLILRB2-His to the surface of *emm3 S. pyogenes*.

2.3.3 Expression of M3 protein at the bacterial surface is necessary for LILRB2 interactions

Having established that blocking of M3 reduces binding of rLILRB2-His to *S. pyogenes*, whether expression of M3 protein is necessary for LILRB2 interaction was tested next. To address this, the binding of rLILRB2-His to the surface of wildtype 950771 and an isogenic *emm3* knockout mutant ($\Delta emm3$) strain was compared by flow cytometry (**Fig 2.2F**). The $\Delta emm3$ strain was previously constructed by the Wessels group, who validated that the $\Delta emm3$ strain did not express M3 by Western blot analysis of cell lysate using anti-M protein serum (Ashbaugh et al. 1998). In these assays, significantly more rLILRB2-His bound to the wildtype *emm3* 950771 strain than the $\Delta emm3$ mutant strain (**Fig 2.2F**). Thus, carriage of the *emm3* gene and the expression of the M3 protein is necessary for the binding of rLILRB2-His to *S. pyogenes*. These data show that the M3 protein is the cognate LILRB2 ligand expressed by *S. pyogenes*.

2.3.4 Bacterial expression of M3 protein is sufficient for LILRB2 binding

To understand whether M3 protein expression at the bacterial surface was solely sufficient to form an interaction with LILRB2, binding of rLILRB2-His to M3 expressing *L. lactis* strains was tested. Wildtype *L. lactis* was transformed with a pOri23.*emm3* vector (**Fig 2.3A**) to express surface M3, pOri23.*emm1* to express surface M1 protein, or pOri23 (empty vector). The pOri23 vectors containing *emm* genes were constructed and sequenced by the Sriskandan group with *emm1* and *emm3* sequences derived from *S. pyogenes* strains MGAS5005 and MGAS315, respectively.

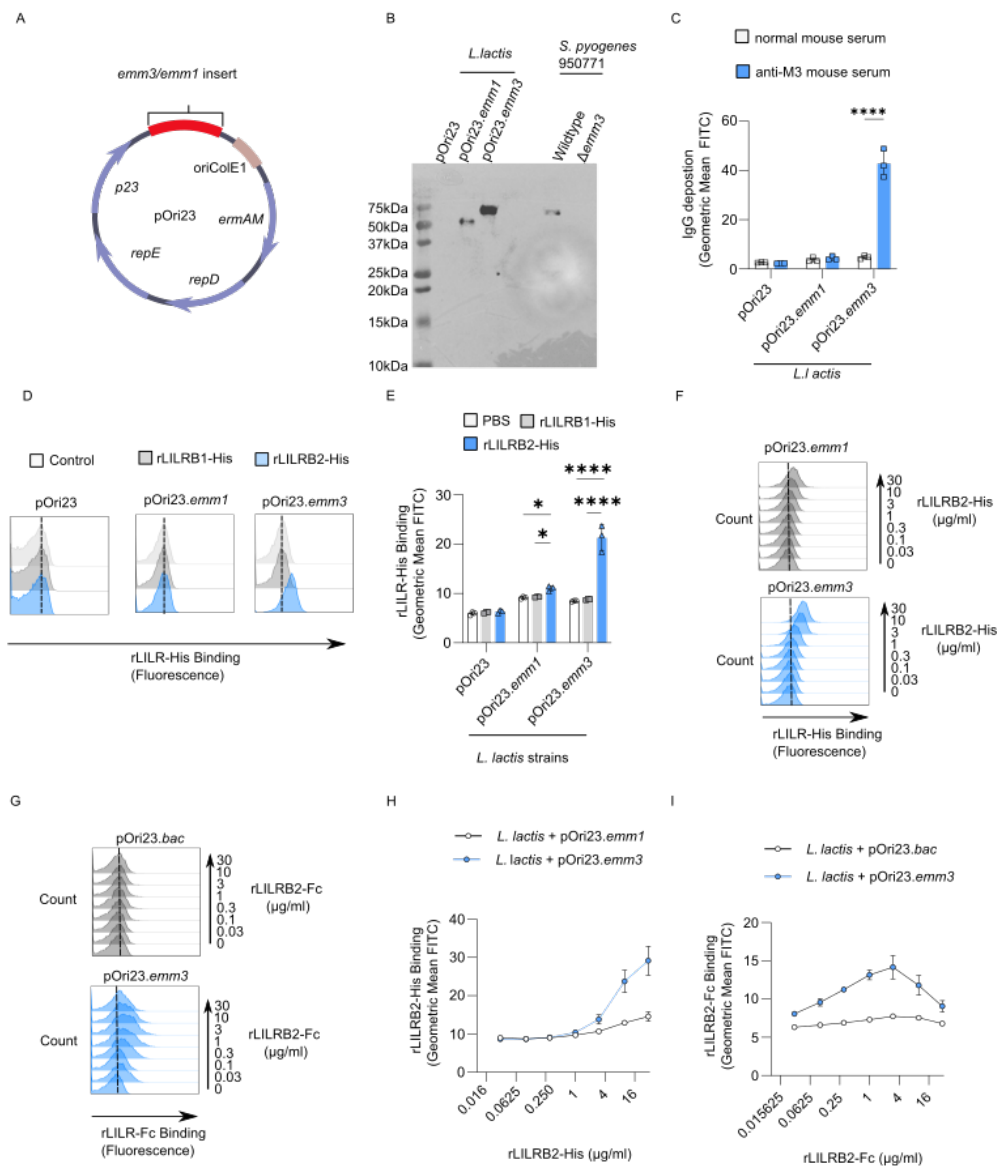


Figure 2.3. Construction of *L. lactis* expressing M3 protein. (A) Schematic of the pOri23 vector used to transform *L. lactis* to express cell-wall anchored M3 or M1 protein. (B) M3 expression in *L. lactis* strains was measured by Western blot. *L. lactis* wildtype (pOri23), M1-expressing (pOri23.*emm1*), and M3-expressing (pOri23.*emm3*) and *S. pyogenes* 950771 wildtype and Δ *emm3* stationary phase bacteria were stained with anti-M3 serum (1:3,000), washed, then binding was measured by staining with goat anti-mouse-FITC (1:10,000). (C) Surface expression of M3 protein on *L. lactis* strains, was detected by incubation in 0.5% (v/v) anti-M3 or non-immune mouse serum, stained with anti-mouse mAb (1:10,000), followed by flow cytometry analysis, $n = 3$ replicates are shown. (D-E) Binding of recombinant LILRB2 (rLILRB2-His) to the surface of *L. lactis* strains was measured by flow cytometry. Stationary phase *L. lactis* strains wildtype (pOri23), M1-expressing (pOri23.*emm1*) or M3-expressing (pOri23.*emm3*) were incubated with rLILRB2-His (10 μ g/ml), rLILRB1-His, or PBS for 30mins, and rLILR-His binding was detected using anti-His FITC (1:50). Representative flow cytometry plot (D) and summary graph (E) of 3 biological replicates. (F-G) Dose-dependent binding of rLILRB2-His (F,H) and rLILRB2-Fc (G,I) to *L. lactis* expressing M3 (pOri23.*emm3*), M1 (pOri23.*emm1*), or β (pOri23.*bac*) was assessed by performing a titration curve. All experiments performed with 3 biological replicates. Mean $\pm$ standard deviation was plotted, and two-way ANOVA was performed to assess statistical significance in (C, E, H and, I) (* $p < 0.05$, **** $p < 0.001$).

To confirm that *L. lactis* + pOri23.*emm3* expressed M3 protein, bacterial lysates were probed for the expression of M3 by Western blot with mouse anti-M3 serum (Fig 2.3B). Notably, the mouse anti-M3 serum exhibited a strong signal in the M3-expressing strain (pOri23.*emm3*) lysate at the predicted molecular weight of M3 (~60 kDa) (Fig 2.3B). Furthermore, *S. pyogenes* 950771 wildtype (*emm3*) exhibited a signal at ~60 kDa and the Δ *emm3* strain did not, indicating that the strain does express the M3 protein as expected. Importantly, the anti-M3 serum did not elicit a signal in the *L. lactis* wildtype strain (pOri23) lysate. However, the anti-M3 serum did elicit a low signal for M1-expressing strain (pOri23.*emm1*) lysate at ~53 kDa, the predicted weight of M1 protein, suggesting cross-reactivity of anti-M3 IgG with M1 proteins (Fig 2.3B), as expected (Smeesters, McMillan, and Sriprakash 2010).

To test whether M3 protein was expressed on the surface of *L. lactis* strains, bacteria were incubated with mouse anti-M3 serum and measured for IgG deposition by flow cytometry (Fig 2.3C). The M3-expressing strain (pOri23.*emm3*) exhibited high levels of IgG deposition, demonstrating surface expression of M3 protein (Fig 2.3C). Wildtype (pOri23) and M1-expressing strains (pOri23.*emm1*) both showed levels of anti-M3 IgG deposition similar to that after incubation in normal mouse serum. Thus, the *L. lactis* + pOri23.*emm3* strain express M3 protein at the bacterial surface.

Next the binding of rLILRB2-His to the panel of validated *L. lactis* strains by flow cytometry was tested (Fig 2.3D-E). rLILRB2-His bound to the M3-expressing *L. lactis* strain significantly more than rLILRB1-His. Importantly, rLILRB2-His did not bind to the *L. lactis* empty vector control. This confirms that the binding of rLILRB2-His was due to M3-expression and not due to alternative *L. lactis* structures. There was some non-specific binding of rLILRB2-His to the M1-expressing strain, which showed a slight significant increase in rLILRB2-His binding compared with rLILRB1-His. However, the increase in binding was minimal and is unlikely to be biologically

significant. Thus, expression of M3 at the bacterial surface is sufficient to bind LILRB2. To show further specificity, *L. lactis* strains were incubated with increasing concentrations of rLILRB2-His (**Fig 2.3F,2.3H**) and rLILRB2-Fc (**Fig 2.3G,2.3I**). Binding of the recombinant proteins was measured by flow cytometry. The binding of rLILRB2-His to the M3-expressing *L. lactis* strain exhibited a dose-dependent response. Incubation with the maximum concentration of rLILRB2-His (30 µg/ml) tested produced the highest levels of rLILRB2-His binding (**Fig 2.3H**). In contrast, rLILRB2-His did not bind to the M1-expressing *L. lactis* strain even at the highest concentration included in the assay (30 µg/ml). The data demonstrated that LILRB2-His binds specifically to bacteria expressing the M3 protein at the cell surface. The assays performed with LILRB2-Fc also revealed similar results (**Fig 2.3I**). Here, the bell-shaped curve is consistent with the Hook effect, whereby high concentrations of an immune molecule impairs its ability to bind its ligand (Self and Cook 1996). LILRB2-Fc allows dimerisation of the receptor which may explain the differences in binding profiles between LILRB2-Fc and LILRB2-His. Importantly, *L. lactis* expressing β protein (pOri23.bac) at the surface (Chapter 3) was used as a negative control because M1 expressed by *L. lactis* (pOri23.emm1) is known to bind IgG via the Fc region. Taken together with the previous data, this provided conclusive evidence that surface expression of M3 protein is both necessary and sufficient for binding of LILRB2 to *S. pyogenes*.

2.3.5 LILRB2 directly binds M3 protein

Having demonstrated that rLILRB2-His binds to M3-expressed on bacterial surfaces, the next investigation explored whether purified rLILRB2 directly bound to purified rM3. This required producing recombinant rM3-His protein to assess direct interactions with rLILRB2.

A *L. lactis* expression system was used to produce recombinant M3 protein with a C-terminal His tag (rM3-His), rather than express rM3-His in *E. coli*. *L. lactis* was chosen because it is a Gram-positive bacterium that likely has post-translational modifications that are more similar to *S. pyogenes* than *E. coli*. Firstly, I designed a pOri23 vector to express rM3-His protein in *L. lactis* (pOri23.emm3-His, **Fig 2.4A**). To ensure rM3-His did not bind to the cell wall and was retained within the cytoplasm, the *emm3* gene was amplified except for the regions encoding the LPxTG cell-wall anchor motif and signal peptide. This would hopefully ensure that the expressed rM3-His could be easily purified from a bacterial pellet to minimise potential protein degradation. A band of the expected size (1.7Kb) corresponding to *emm3* was gained following PCR. The DNA at this band was excised from the gel, digested with *Bam*HI/*Sal*I and ligated into pOri23 vector that had previously been digested with *Bam*HI/*Sal*I. The ligation reactions were subsequently transformed into *E. coli* and cultured on LB agar plates containing erythromycin to select for clones carrying pOri23 vectors.

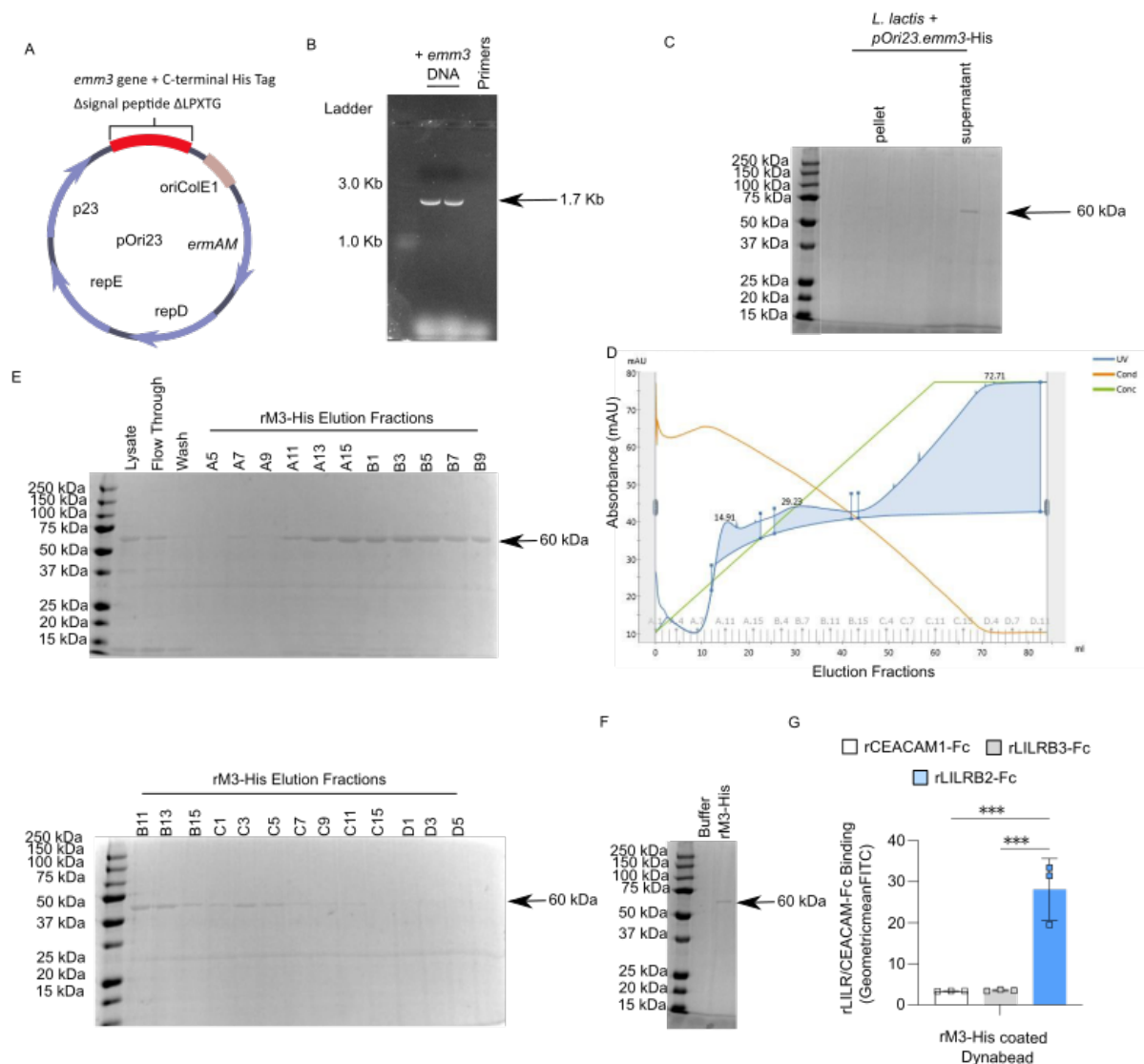


Figure 2.4. LILRB2 directly binds M3-His protein. (A) Schematic of the pOri23 vector used to transform *L. lactis* to express recombinant M3-His (rM3-His). (B) Analysis of pOri23.*emm3*-His vector by agarose gel. PCR was performed across pOri23 multiple cloning site, to check for insertion of *emm3* gene. (C) rM3-His expression by SDS-PAGE. *L. lactis* clones transformed with pOri23.*emm3*-His were cultured overnight, then the supernatant and pellet were mixed 1:1 with sample buffer, and protein expression was visualised by Coomassie blue staining. (D-E) Purification of recombinant rM3-His by ATKA™ protein purification system. *L. lactis* supernatant was passed through His-trap columns, which were eluted using an imidazole gradient (10-500 mM). The absorbance of eluted fractions (D) was used to determine the fractions to be tested for the presence of recombinant protein by Coomassie stain (E). (F) SDS-PAGE assessing purity of rM3-His from isolated fractions (G) rLILRB2-Fc binding to rM3-His was assessed by flow cytometry. rM3-His coated Dynabeads were incubated with rLILRB2-Fc, rLILRB3-Fc, or rCEACAM1-Fc, then washed, and stained for binding of Fc-tagged proteins using goat anti-human-IgG-FITC (1:1,000). Experiments were performed with 3 biological replicates. Mean $\pm$ standard deviation was plotted, and one-way ANOVA (G) was performed to assess statistical significance (***) $p < 0.005$.

The pOri23 vector constructed was tested by PCR to confirm insertion of the *emm3* gene (**Fig 2.4B**). Following sequencing validation, the assembled vector pOri23.*emm3*-His was transformed into *L. lactis*. Next, to assess transformants for rM3-His protein expression and subcellular location, the total protein expression in pellets and supernatants of *L. lactis* + pOri23.*emm3*-His transformants was measured by SDS-PAGE (**Fig 2.4C**). Surprisingly, more rM3-His (predicted size ~ 60 kDa) was observed in the supernatant of the *L. lactis* + pOri23.*emm3*-His strain compared to the pellet. This suggested that rM3-His was still being secreted into the supernatant despite deletion of the signal peptide sequence (**Fig 2.4C**). One explanation is that there was potential leakage of rM3-His protein into the supernatant due to high levels of expression, and differences in the composition between *L. lactis* and *S. pyogenes* cell membrane and cell wall.

Next, affinity chromatography was used to purify rM3-His from the supernatant of *L. lactis*. To initially concentrate the supernatant, filter columns were used to remove molecules <10 kDa from the supernatant. To further purify the supernatant, the supernatant was washed several times in PBS. This involved repeatedly diluting supernatant in PBS then centrifuging to remove PBS and impurities. The purified supernatant was supplemented with 10 mM imidazole and loaded onto a His-trap column. It was expected that the rM3-His proteins would bind to the His-trap column and then could be subsequently eluted using the ATKA™ protein purification system and increasing concentrations of imidazole. Indeed, rM3-His was eluted from the His-trap column by passing a gradient of imidazole (10 mM-500 mM) over the His-trap column. The absorbance of elution fractions was monitored throughout the purification process to help identify those that containing rM3-His protein (**Fig 2.4D**). There were multiple peak absorbances at 14.91 ml, 29.23 ml, and 72.71 ml; therefore, determining the elution fractions containing rM3-His protein was challenging. Therefore, samples were taken from every second elution fraction and measured protein content by SDS-PAGE (**Fig 2.4E**). Elution fractions A11 (top gel) to C5 (bottom gel) contained high concentrations of a protein of the expected size of rM3-His (60 kDa). Therefore, these fractions were pooled and concentrated using a 10 kDa filter column and purity was tested by SDS-PAGE (**Fig 2.4F**).

Having successfully expressed and purified rM3-His protein from the *L. lactis* expression system, the direct interaction of rLILRB2-Fc with M3 protein was tested. Dynabeads were coated with M3-His and incubated with rLILRB2-Fc, rLILRB3-Fc or rCEACAM1-Fc and binding of recombinant Fc-proteins was measured by flow cytometry (**Fig 2.4G**). rLILRB3-Fc and rCEACAM1-Fc were used as negative controls instead of LILRB1-Fc because rLILRB1-Fc was yet to be constructed. rLILRB2-Fc bound to M3-His coated Dynabeads significantly more than rLILRB3-Fc or rCEACAM1-Fc (**Fig 2.4G**). rLILRB3-Fc and rCEACAM1-Fc did not bind M3-coated Dynabeads, demonstrating that rLILRB2-Fc specifically binds to M3 protein via the LILRB2 domain, and not the Fc-tag. Thus, LILRB2 directly binds to M3 protein.

2.3.6 M3-mediated engagement of LILRB2 does not enhance bacterial survival in whole blood

Having established that the M3 protein of *S. pyogenes* is a ligand for LILRB2, the next assays sought to understand whether *S. pyogenes* utilises M3 to engage LILRB2 for immune evasion purposes. As previously discussed, LILRB2 modulates immune cells present in the blood by suppressing activation and anti-microbial effector functions (Lewis Marffy and McCarthy 2020). Thus, the hypothesis was that M3-dependent engagement of LILRB2 by *S. pyogenes* suppresses immune cells as an immune evasion strategy.

To address this hypothesis, assays were performed blocking the binding of *S. pyogenes* to LILRB2 receptors on immune cells to determine whether this would enhance their survival in whole blood. In these assays, *S. pyogenes* (strain, 950771) inocula were preincubated with rLILRB2-His, rLILRB1-His, or PBS for 30 mins, and washed to remove unbound rLILR-His. For 950771 incubated in rLILRB2-His, it was expected that rLILRB2-His would bind M3 protein and block its capacity to bind LILRB2 expressed on immune cells. In contrast, rLILRB1-His would not block the interaction of M3 and LILRB2. These inocula were used to infect whole blood and bacterial growth was measured after 3 hours by CFU counts (**Fig 2.5A**). The multiplication factor of *S. pyogenes* (bacterial growth) was derived by normalising CFU counts after 3 hours incubation in blood with the initial inoculum. There was no significant difference in growth of the 950771 strains that had been preincubated with rLILRB2-His compared with rLILRB1-His or PBS (**Fig 5A**). These results suggested that *S. pyogenes* engagement with immune cells via the LILRB2 receptor does not promote *S. pyogenes* survival in whole blood under these experimental settings. This could be due to redundancy in *S. pyogenes* immune evasion mechanisms, as *S. pyogenes* expresses a plethora of virulence factors that promote immune evasion (Smeesters, McMillan, and Sriprakash 2010). Thus, detectable differences in bacterial survival through blocking of a single interaction such as M3-LILRB2 may not be sufficient.

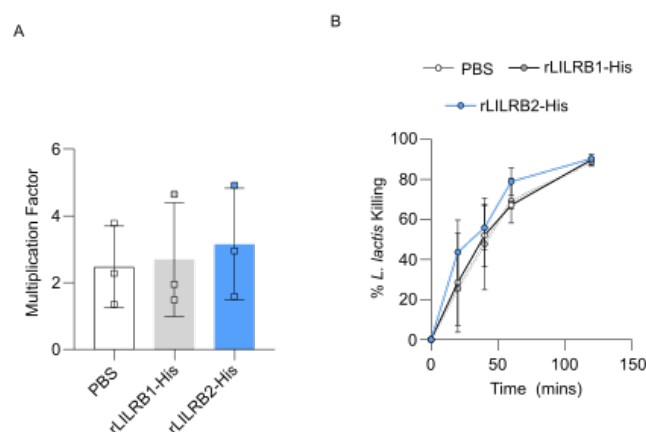


Figure 2.5. Blocking *S. pyogenes* and *L. lactis* interaction with LILRB2 does not enhance bacterial survival in whole blood. (A) *S. pyogenes* survival in whole blood after incubation with recombinant LILR. Midlog phase *S. pyogenes* strains were incubated with PBS, rLILRB1-His (30 µg/ml), or rLILRB2-His (30 µg/ml) for 30 mins, washed, then samples were inoculated into human whole blood and incubated at 37°C for 3 hours. CFU count was measured, and the multiplication factor in whole blood was determined. **(B)** *L. lactis* survival in whole blood after incubation with rLILRB1-His or rLILRB2-His at several timepoints. Method was the same as in **(A)** although CFU counts were taken at multiple timepoints: 30 mins, 45 mins, 60 mins and 120 mins. Mean ± standard deviation was plotted, and one-way ANOVA was performed to assess statistical significance

To reduce the potential role of other *S. pyogenes* immune evasion molecules, the next investigation determined whether blocking of LILRB2 engagement by M3 enhanced the killing of *L. lactis* in whole blood. *L. lactis* does not express virulence factors providing a useful model to interrogate bacterial immune evasion. M3-expressing *L. lactis* (pOri23.emm3) was preincubated with rLILRB2-His, rLILRB1-His, or PBS and whole blood killing was measured by CFU counts at 20 mins, 40 mins, 60 mins, and 120 mins (**Fig 2.5B**). There was no significant difference in the killing of M3-expressing *L. lactis* following preincubation with rLILRB2-His, compared with rLILRB1-His or PBS for any of the time points. Thus, M3 engagement of LILRB2 did not enhance immune evasion of *L. lactis* under these experimental settings. However, high variation in the data would have prevented determining significant differences, suggesting the assay must be optimised further to be sure of conclusions. Furthermore, the minimal differences detected in survival may be due to incomplete saturation of LILRB2 binding sites. *L. lactis* was preincubated with 30 µg/ml rLILRB2-His; however, a previous titration assay suggested that higher concentrations of rLILRB2-His may be required to fully saturate LILRB2 binding sites (**Fig 2.3H**). However, time constraints prevented further optimisation of this assay or the testing of alternative neutrophil killing assays.

In conclusion, these preliminary experiments did not support the hypothesis that *S. pyogenes* engages LILRB2 on immune cells via M3 for immune evasion purposes. However, the whole blood assays used to determine immune evasion need to be further optimised before definitive conclusions can be made. As whole blood assays have a high level of noise to signal ratio, due to the huge number of potential interactions of bacteria with a range of immune-related factors, it would be useful to investigate the role of M3-LILRB2 engagement using different purified components. For example, purified neutrophils or monocyte-derived macrophages assays may be more sensitive for interrogating LILRB2-mediated immune evasion.

2.3.7 Soluble LILRB2 may block M3-mediated bacterial adhesion

To understand alternative functional consequences of M3-LILRB2 engagement, the next assays investigated whether rLILRB2-His may inhibit bacterial adhesion. Adhesion is an essential step for pathogenic bacteria to first

colonise host epithelial surfaces prior to subsequent tissue invasion. Most LILR genes contain a stop codon within the 5' of the transmembrane encoding exon, which through mRNA splicing, can generate soluble LILR isoforms (Jones et al. 2009). Thus, it is possible that soluble LILRB2 (sLILRB2) is expressed in the human body. Indeed, sLILRB2 has been detected in the supernatant of dendritic cells (Beinhauer et al. 2004). Thus, secretion of LILRB2 may be a potential mechanism the host employs to inhibit the adhesion of M3-expressing *S. pyogenes* to host structures via M3-dependent interactions. Therefore, the hypothesis tested was that M3-mediated bacterial adherence to collagen I and collagen IV can be blocked by rLILRB2-His. To address this hypothesis, a *L. lactis* adhesion model was designed. This methodology was selected because the *L. lactis* model enables specific interrogation of M3-mediated adherence, whereas *S. pyogenes* expresses several proteins that bind collagen including ancillary protein 1 (AP1) and collagen-binding protein of group A (CpA) that could confound results (Falugi et al. 2008; Kreikemeyer et al. 2005). *L. lactis* strains were incubated on plates coated with BSA, collagen I, or collagen IV, vigorously washed, then stained with crystal violet, and adherent bacteria were measured by microplate reader. The adhesion model was validated by confirming that M3-expressing *L. lactis* specifically adhered to collagen I and collagen IV (**Fig 2.6A**). The M3-expressing *L. lactis* showed significantly greater adherence to collagen I and collagen IV compared with BSA (**Fig 2.6A**). Importantly, M1-expressing *L. lactis* and the control *L. lactis* strain exhibited no significant difference in adhesion to collagen I or collagen IV (**Fig 2.6A**). Thus, these data validated the use of the *L. lactis* adhesion model to determine the functional consequences of LILRB2-mediated inhibition of M3 binding to collagen.

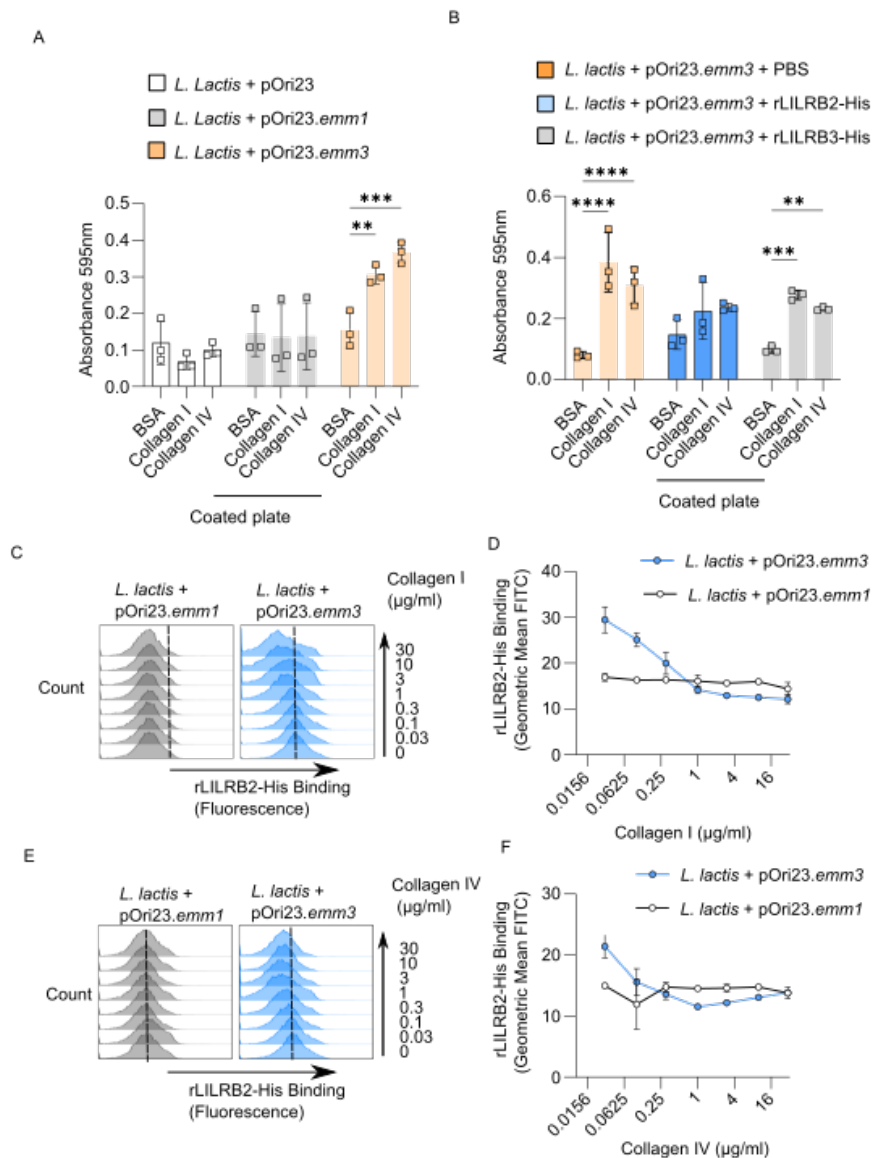


Figure 2.6. Recombinant LILRB2 inhibits M3-expressing bacterial adhesion to collagen I and IV. (A-D) (A) Bacterial adhesion was assessed by binding of bacteria to collagen-coated plates. Stationary phase *L. lactis* strains wildtype (pOri23), M1-expressing (pOri23.emm1) or M3-expressing (pOri23.emm3) were incubated on BSA or collagen I or collagen IV coated plates, plates were washed several times with PBS, then stained with crystal violet (0.5%) to measure adherent bacteria. (B) LILRB2-mediated inhibition of bacterial adhesion to collagen I or collagen IV was measured. *L. lactis* strains were pre-incubated with PBS, rLILRB2-His (10 μg/ml), or LILRB3-His (10 μg/ml) and then adherence to collagen-coated plates was assessed using the same method as (A). (C-F) Collagen-mediated inhibition of LILRB2 binding to *L. lactis* strains. *L. lactis* strains were incubated with increasing concentrations of collagen I (C-D) or collagen IV (E-F) and rLILRB2-His surface binding was measured by flow cytometry. Representative flow cytometry plot (C,E) and summary graph (D,F) of 3 biological replicates. Mean ± standard deviation was plotted, and two-way ANOVA was performed to assess statistical significance in (A-B) (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$).

Next, whether rLILRB2-His inhibits M3-mediated bacterial adhesion to collagen I and collagen IV was determined (**Fig 2.6B**). Previous assays (not shown) had demonstrated that rLILRB1-His and rLILRB3-His did not bind M3-expressing *L. lactis* (pOri23.*emm3*), and thus, either rLILR-His could be used as a protein control. rLILRB3-His was chosen as a protein control in favour of rLILRB1-His, because rLILRB3-Fc, and not rLILRB1-Fc, was already constructed if additional/alternative adhesion assays were required. M3-expressing *L. lactis* (pOri23.*emm3*) was preincubated with 10 µg/ml of rLILRB2-His, rLILRB3-His, or PBS, washed to remove unbound rLILR-His, and incubated on either BSA, collagen I or collagen IV coated plates. M3-expressing *L. lactis* preincubated in PBS had significantly increased adherence to collagen I or collagen IV compared with BSA control (**Fig 2.6B**). However, preincubation with rLILRB2-His (10 µg/ml) inhibited M3-expressing *L. lactis* adherence to collagen I or collagen IV to levels comparable to BSA (**Fig 2.6B**). Importantly, preincubation with rLILRB3-His, which acted as a protein control did not inhibit M3-expressing *L. lactis* adherence to collagen I or collagen IV (**Fig 2.6B**). M3-expressing *L. lactis* preincubated with rLILRB3-His and PBS, exhibited a similar collagen adhesion profile (**Fig 2.6B**). These data demonstrated that rLILRB2-His specifically inhibits M3-mediated *L. lactis* adhesion to collagen I or collagen IV.

Finally, the specificity of the rLILRB2-His interaction with M3-expressing by *L. lactis* by competitively inhibiting the interaction using soluble collagen I and collagen IV was determined. M1- and M3-expressing *L. lactis* strains were pre-incubated with increasing concentrations of collagen I (**Fig 2.6C-D**) or collagen IV (**Fig 2.6E-F**) and the binding of rLILRB2-His was measured by flow cytometry. Preincubation of M3-expressing *L. lactis* with either collagen I or collagen IV reduced rLILRB2-His binding to M3-expressing *L. lactis* in a dose dependent manner (**Fig 2.6C-F**). In support of previous collagen blocking assays using *emm3 S. pyogenes* 950771, (**Fig 2.6E-F**), collagen IV was more effective at blocking rLILRB2-His binding to M3-expressing *L. lactis* (**Fig 2.6D,2.6F**). The minimum threshold inhibitory concentration to block LILRB2-His binding to levels similar to M1-expressing *L. lactis* was 0.03 µg/ml for collagen I (**Fig 2.6D**) and 0.01 µg/ml for collagen IV (**Fig 2.6F**). Furthermore, rLILRB2-His did not bind to M1-expressing *L. lactis* (**Fig 2.6C-F**). Thus, these data demonstrated that collagen I and collagen IV specifically inhibit LILRB2-M3 binding. These data supported the hypothesis that soluble LILRB2 inhibits M3-mediated bacterial adhesion to collagen I and collagen IV.

2.4 Discussion

The main finding of this Chapter was the discovery that M3 protein from *S. pyogenes* is a ligand for the human inhibitory immune receptor LILRB2. Exploring the interaction of bacterial pathogens and inhibitory immune receptors enables improved understanding of bacterial pathogenesis and host responses to bacterial infections (Avondt, Sorge, and Meyaard 2015), both key areas for developing novel therapeutics for prevention and/or treatment of bacterial diseases. The M3 protein of *S. pyogenes* is the first LILRB2 ligand identified from a human bacterial pathogen, which now provides opportunities to dissect the role of LILRB2 targeting in bacterial immune evasion. In similar fashion, the RIFIN molecules of the protozoan parasite *P. falciparum* are known to directly interact with LILRB2 (Sakoguchi et al. 2021). As LILRB2 is an important inhibitory receptor that suppresses effector responses of innate immune cells (Singh et al. 2022; Carlin et al. 2009), the targeting of LILRB2 likely represents an important mechanism for *S. pyogenes*, *P. falciparum* and other pathogens to evade immune responses.

In this Chapter, M3 protein of *S. pyogenes* was demonstrated to be a ligand for LILRB2 through a series of complementary assays. Initially, expression of M3 protein on bacterial surfaces was shown to be required for rLILRB2-His binding, and subsequently that rM3-His directly interacted with rLILRB2-Fc. The M3 protein is an important virulence factor in *S. pyogenes*, as revealed by mice studies demonstrating that expression of M3 is essential for virulence during invasive disease (Herrera et al. 2017). Beyond LILRB2, the M3 protein also interacts with numerous components of the human system including collagen IV, fibronectin, fibrinogen, albumin, complement factors (C3b/iC3b), and IgG3 (**Fig 2.2A**) (Smeesters, McMillan, and Sriprakash 2010). The interaction of M3 protein with these host factors is proposed to increase virulence through enhancing *S. pyogenes* adhesion. For example, *S. pyogenes* binds to soluble albumin and fibrinogen via M3 protein resulting in enhanced bacterial adhesion to the lower respiratory tract of mice and increased virulence (Herrera et al. 2017). Thus, characterisation of LILRB2 as a new human ligand for M3 is important because it may explain the key role M3 protein plays in virulence and may reveal a novel immune evasion strategy, both of which are important for aiding development of novel therapeutics.

Through these investigations, it was demonstrated that a wide repertoire of clinical *emm3 S. pyogenes* strains bind LILRB2 but that there were high levels of heterogeneity in LILRB2 binding capacity. A screen of a panel of clinical *emm3* isolates for LILRB2 binding showed that all but one strain interacts with LILRB2. The difference in LILRB2 binding between strains may be reflected by different expression levels of M3 protein at the *S. pyogenes* surface. This could result if mutations exist in transcriptional or translational regulation genes; for example, mutations in the *covRS* two-step regulatory gene have been shown to increase levels of M protein expression (Bao et al. 2015). Future assays should test surface M3 expression of *S. pyogenes* strains using anti-M3 serum and assess whether LILRB2 binding is correlated with IgG deposition of anti-M3 serum. Alternatively, differences in LILRB2 binding to *emm3 S. pyogenes* strains may be explained by variations in the N-terminal hypervariable region of M3 proteins. A study analysing the *emm*-types of 1064 invasive *S. pyogenes* strains in the USA, revealed

8 subtypes of M3 protein (Li et al. 2003). Subtypes were defined as having more than 1 residue change in the coding region within the 50 N-terminal residues of translated M3 proteins. Therefore, the panel of *emm3* strains tested may express different M3 protein subtypes that have differences in the N-terminal domain altering LILRB2 binding. To determine if M3-subtype was important for LILRB2 binding, recombinant M3 subtypes should be constructed and test the binding to LILRB2-Fc. Importantly, identification of M3-subtypes that bind LILRB2 more readily could also identify M3 protein residues important for LILRB2 interaction and/or assist in locating the LILRB2 binding domain within M3.

Defining the LILRB2-M3 binding interface is important to produce the necessary tools to fully interrogate the functional consequences of the interaction for immune evasion. The data presented here show that collagen acts as a competitive inhibitor of LILRB2-M3 interaction. This suggests that the location of the LILRB2 binding domain(s) and collagen binding domain on M3 protein overlap. M proteins that bind collagen IV express a PARF (peptide associated with rheumatic fever) motif - (A/T/E)XYLXX(L/F)N - located in the N-terminal region for collagen IV binding (Reißmann et al. 2012). Thus, the LILRB2 binding domain is likely located in the N terminal domain of the M3 protein.

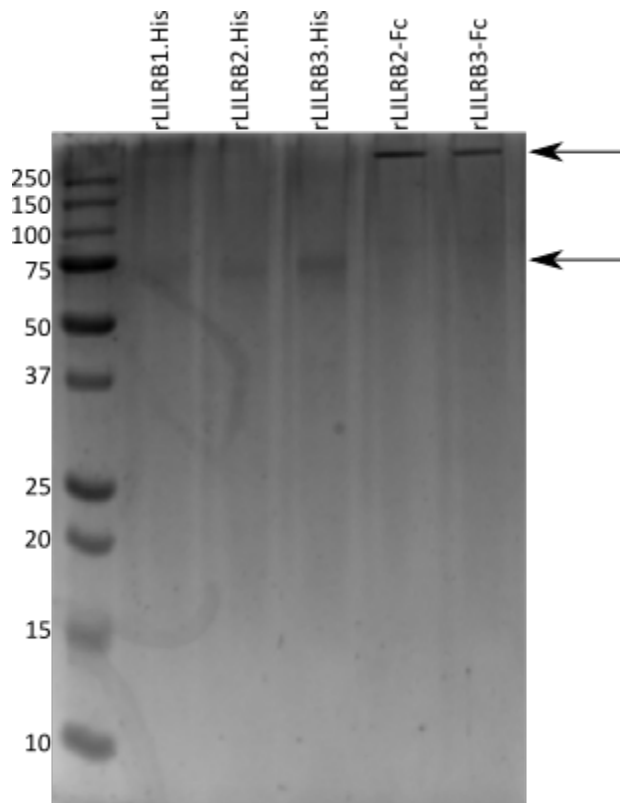
The preliminary experiments revealed that M3 engagement of LILRB2 is not necessary for *S. pyogenes* immune evasion in whole blood. Blocking of *S. pyogenes* interaction with LILRB2 using rLILRB2-His did not abrogate immune evasion capacity of *emm3 S. pyogenes* in whole blood. There are several potential explanations for this. Firstly, there may be redundancies in M3-mediated immune evasion, as M3 protein binds to multiple host ligands that may play redundant roles for promoting immune evasion during invasive disease (Happonen et al. 2019). Secondly, the M3 proteins expressed on *S. pyogenes* may not be saturated with rLILRB2-His after 3 hours of growth. As shown by our titration curve, 30 µg/ml of rLILRB2-His potentially does not completely saturate LILRB2-binding sites on surface-expressed M3 because we did not observe a plateau unlike with rLILRB2-Fc. Therefore, higher concentrations of rLILRB2-His may be required to fully saturate LILRB2-binding domains on *S. pyogenes* and measure observable difference in *S. pyogenes* growth. Furthermore, as *S. pyogenes* replicates over 3 hours, the levels of rLILRB2-His saturation would decrease. In future assays, whether the LILRB2-M3 interaction may be more effectively blocked by spiking whole blood with a LILRB2 mAb to block LILRB2 engagement should be tested. However, this would require knowledge of the M3 interface on LILRB2 and the identification of a blocking mAb in biochemical assays. Similarly, blocking the LILRB2 interaction with M3 in M3-expressing *L. lactis* did not impact bacterial killing. As LILRB2 is expressed on a range of immune cells (Zhang et al. 2017), additional assays that assess the impact of the M3-LILRB2 interaction on specific cellular functions is required. It is possible that using whole blood from healthy patients may not be the appropriate model for determining the role of LILRB2 engagement by bacteria during infection, especially if LILRB2 is over-expressed during infection. There are some data that supports this concept, as septic shock patients exhibit significantly higher levels of LILRB2 protein expression on monocytes at day 3 compared with healthy controls (Yu et al. 2018). Interestingly, LILRB2 protein expression in monocytes primed with LPS *ex vivo* is initially downregulated (<24 hours) and subsequently upregulated (>48 hours) (Yu et al. 2018). Thus, these data may indicate that LILRB2 expression is enhanced on immune cells during later stages of invasive disease, where it could be targeted by

pathogens for immune evasion purposes. Thus, in future assays it would be interesting to compare M3-expressing *S. pyogenes* and *L. lactis* bacterial survival in monocytes primed for 1 hour, 24 hours, or 48 hours. To specifically determine whether LILRB2 interaction was involved in increased survival, it would be useful to also block LILRB2 interactions using an antagonistic mAb for each time point.

Finally, recombinant LILRB2 inhibits *S. pyogenes* adhesion to collagen surfaces suggesting a potential anti-microbial host mechanism. Preincubation of M3-expressing *L. lactis* with rLILRB2-His inhibited bacterial adhesion to collagen I and IV coated plates. Both *in vitro* and *in vivo* assays have demonstrated that *S. pyogenes* utilises M protein to bind collagen and enhance colonisation (Dinkla et al. 2003). Thus, secretion of LILRB2 may be a useful anti-microbial mechanism the host employs to inhibit *S. pyogenes* colonisation. As previously discussed, dendritic cells secrete soluble LILRB2 and whilst secretion in other innate immune cells has not been studied, alternative mRNA splicing suggest these immune cells have the capacity to produce soluble LILRB2 (Beinhauer et al. 2004; Jones et al. 2009). Secreted LILRB2 may also serve to enhance inflammatory responses to *S. pyogenes* by preventing LILRB2-mediated inhibitory signalling. Indeed, secretion of inhibitory receptors has been shown to enhance inflammatory responses. For example, NK cells secrete CEACAM1 resulting in enhance NK activation; a process that is downregulate in patients with TAP2 autoimmune disorders to compensate for hyper immune activity (Markel et al. 2004). Thus, LILRB2-M3 interaction may also be utilised by the host to inhibit colonisation and promote inflammatory responses to *S. pyogenes*.

In conclusion, the M3 protein of *S. pyogenes* was identified as the first bacterial ligand of LILRB2. The ligand is expressed by *emm3* *S. pyogenes* strains that are often associated with severe invasive disease. Whilst preliminary assays did not reveal the functional consequences of the interaction for immune evasion, further investigation is required to reveal the molecular details and functions of this interaction in bacterial immune evasion. Furthermore, the potential role of secreted LILRB2 during *S. pyogenes* infection as a host anti-microbial mechanism requires further investigation.

2.5 Supplementary figures



Supplementary Figure 2.1: Pure recombinant LILR-His and LILR-Fc. Purity of recombinant LILR was assessed by SDS-PAGE. Recombinant LILR samples show single bands at ~75kDa (rLILR.His, bottom arrow) and ~250kDa (rLILR-Fc, top arrow).

Chapter 3. *S. agalactiae* engages the inhibitory LILRB3 receptor via β protein

3.1 Introduction

In this Chapter, whether *S. agalactiae* expresses a ligand that interacts with the inhibitory immune receptor LILRB3 to suppress immune functions and enhance immune evasion was investigated.

Neutrophils are the key immune cell responsible for clearance of extracellular bacteria through potent effector mechanisms. However, the release of proteases can have damaging effects on tissues. Therefore, neutrophils must be tightly regulated to limit inappropriate activation, tissue damage and inflammation. Neutrophils, like other immune cells, are tightly regulated by activating and inhibitory receptors expressed on the cell surface that enables them to sense their external environment. It is through this receptor sensing that neutrophils can mount robust responses to pathogenic threats and to sense environments in which cellular activation is switched off to prevent host damage. Neutrophils express several inhibitory receptors to regulate their activation, including LILRB1, LILRB2, LILRB3, CEACAM1, SIRL1, and LAIR1 (Avondt, Sorge, and Meyaard 2015). Several human pathogens are known to directly interact with inhibitory immune receptors to promote their immune evasion.

LILRB3 is an inhibitory immune receptor is expressed on the surface of circulating neutrophils. It is also expressed by myeloid cells such as eosinophils, basophils, monocytes, and macrophages, and also osteoclasts and dendritic cells (Abdallah et al. 2021). LILRB3 is expressed both within granules and at the cell surface (Lewis Marffy and McCarthy 2020), thus, LILRB3 can modulate both the threshold and amplitude of neutrophil activation and immune effector functions. For example, LILRB3 suppresses neutrophil Fc receptor-mediated functions such as production of reactive oxygen species, phagocytic uptake, and microbial killing (Zhao et al. 2020). Therefore, LILRB3 presents an important target that if bacterial pathogens exploit, can suppress neutrophil activation and effector functions and enhance bacterial survival. The upregulation of LILRB3 on macrophages during sepsis and the observation that LILRB3 blockade decreases susceptibility to sepsis in a mouse sepsis model (Ming et al. 2019) together suggest that LILRB3 may be directly targeted by pathogens to evade the immune system. Thus, exploiting LILRB3-mediated suppression may be an effective strategy for a pathogen to subvert immune responses and promote their immune evasion.

LILRB3 contains key structural features that are important for inducing inhibitory signalling. LILRB3 is composed of an extracellular domain containing four Ig-like domains, a transmembrane domain, and a long cytoplasmic tail containing four ITIM domains through which it propagates inhibitory signalling (Billadeau and Leibson 2002). Upon ligation of the Ig-like domains, the ITIM domains are phosphorylated, enabling recruitment of phosphatases SHP-1 and SHP-2 (Mori et al. 2008). Recruitment of these phosphatases results in dephosphorylation of ITAM domains of nearby activating receptors, thus attenuating activating signalling in immune cells. For example, LILRB3 suppresses FcεRI- and LILRA2- mediated release of effector molecules such as histamine, IL-4 generation, and cysteinyl leukotrienes in basophils (Yu et al. 2018). Furthermore, LILRB3 ligation in peripheral blood mononuclear cells has been shown to modulate T cell activation and proliferation (Yeboah et al. 2020). Thus, LILRB3 modulates global immune responses, whose effects are not solely restricted to LILRB3-expressing myeloid cells.

The only ligand known to bind LILRB3 is APOE4, a host protein important for immune regulation, lipid metabolism, and neurology in glial cells (Zhou et al. 2023). The binding of APOE4 to LILRB3 was located to the D2 domain. Surprisingly, APOE4-mediated engagement of LILRB3 expressed on microglia cells upregulates expression of pro-inflammatory genes (Zhou et al. 2023). Thus, whilst LILRB3 is an inhibitory receptor that suppresses neutrophil effector functions (Zhao et al. 2020), the suppressive function appears to be dependent on cell type and/or ligand engagement. No other ligands of LILRB3 are known. Importantly, LILRB3 is also paired with the activating receptor LILRA6, but this is not expressed on neutrophils (Zhao et al. 2020). The recognition of APOE4 as an endogenous ligand of LILRB3 now paves the way to better understand the function of LILRB3 in the human immune system.

It is currently unknown whether bacterial pathogens directly interact with LILRB3 for immune evasion purposes. Studies involving PIR-B, the mouse ortholog for LILRB1-6 receptors, suggested that *S. aureus* binds to PIR-B-expressing cell lines, although, the ligand and functional consequences were not elucidated (Nakayama et al. 2007). In an additional study, PIR-B deficient mice were more susceptible to *S. enterica* infection (Yu et al. 2018). However, whether LILRB3, which has important structural differences to PIR-B (PIR-B contains 6 Ig-like domains in its extracellular domain as opposed to 4) interacts with bacteria for immune evasion is unknown. Other inhibitory LILR are known to be exploited by human pathogens for immune evasion purposes. For example, the erythrocyte-invading parasite *P. falciparum* express a family of RIFIN on erythrocyte cell surfaces that interact with LILRB1 and LILRB2 (Sakoguchi et al. 2021; Saito et al. 2017). RIFIN engagement of LILRB1 dampens NK responses and enhances survival of *P. falciparum* in erythrocytes (Saito et al. 2017). In contrast, the functions of LILRB2 and RIFIN interactions have not yet been reported.

A preliminary screen performed by the McCarthy group (unpublished) indicated a putative interaction of *S. agalactiae* with LILRB3. *S. agalactiae* causes a range of invasive diseases that are a large global health burden, particularly in infants where it causes pneumonia, sepsis, and meningitis (Shabayek and Spellerberg 2018). Indeed, *S. agalactiae* is responsible for ~150,000 still births and neonatal deaths worldwide each year (Seale et al. 2017). There is currently no vaccine to protect against *S. agalactiae* diseases, and efforts have been hampered, in part, by the high diversity of *S. agalactiae*, with 10 serotypes expressing different polysaccharide

capsules and proteins (Ia, Ib, II-IX) (Yu et al. 2018). Of note, only a subset of serotypes is responsible for invasive disease in infants with 97% of invasive isolates being serotype Ia, Ib, II, III and V (Carreras-Abad et al. 2020). However, the high genomic diversity of the *S. agalactiae* population makes identifying a potential LILRB3 ligand challenging.

3.1.1 Hypothesis and Aims

The hypothesis was that *S. agalactiae* expresses a ligand(s) that directly engages LILRB3 for immune evasion thereby promoting bacterial survival. The aims were to (1) identify the *S. agalactiae* ligand that binds LILRB3, (2) characterise the ligand-LILRB3 binding interface, (3) determine the functional consequences of LILRB3 engagement for *S. agalactiae* immune evasion.

3.2 Materials and methods

3.2.1 Bacterial strains and growth conditions

S. agalactiae (wild-type, Δbac , and Δbac + pLZ.*bac*) and the parental *L. lactis* MG1363 strain have been previously described (Areschoug et al. 2002; Wegmann et al. 2007). *S. agalactiae* strains (**Table 3.1**) were cultured at 37°C in Todd Hewitt broth (THY). For growth of the A909 Δbac + pLZ.*bac* strain, THY was supplemented with 70 µg/ml spectinomycin. *L. lactis* strains (**Table 3.2**) were cultured at 30°C in M17 broth supplemented with 0.5% (w/v) glucose and 0.5% (w/v) lactose (GM17) and strains with pOri23 were cultured in GM17 supplemented with 5 µg/ml erythromycin. *E. coli* strains (**Table 3.3**) were cultured in Lysogeny broth (LB) at 37°C with shaking. For strains containing pRSET and pOri23 vectors, LB was supplemented with 100 µg/ml ampicillin and 5 µg/ml erythromycin, respectively.

Strain	Vector	Notes	Reference
<i>Streptococcus agalactiae</i>			
A909		Serotype Ia; <i>bac</i> positive	(Areschoug, Linse, et al. 2002)
515		Serotype Ia; <i>bac</i> negative	(Banerjee et al. 2011)

COH1		Serotype III; <i>bac</i> negative	ATCC #BAA-1176
H36B		Serotype Ib; <i>bac</i> positive	ATCC #BAA-1174
NEM316		Serotype III; <i>bac</i> positive	(van Sorge et al. 2021)
NCTC1084		Serotype V; <i>bac</i> negative	ATCC #49447
SB20		Serotype III; <i>bac</i> positive	(Banerjee et al. 2011)
BS29		Serotype II; <i>bac</i> positive; invasive isolate	(van Sorge et al. 2021)
BM110		Serotype III; <i>bac</i> negative	(Bateman, Eddy, and Chothia 1996)
BS39		Serotype Ia; <i>bac</i> negative; invasive isolate	(van Sorge et al. 2021)
SB35		<i>bac</i> positive	(Bateman, Eddy, and Chothia 1996)
BS22		Serotype Ib; <i>bac</i> ositive	(Banerjee et al. 2011)
BS26		<i>bac</i> positive	(Banerjee et al. 2011)
SBL3066		Serotype V; <i>bac</i> positive; invasive isolate	(van Sorge et al. 2021)
18RS21		Serotype II; <i>bac</i> negative	ATCC #BAA1175
A909		Serotype Ia; <i>bac</i> positive	(Areschoug, Linse, et al. 2002)

A909 Δbca		A909 with <i>bca</i> gene deleted	(Areschoug, Stålhammar-Carlemalm, et al. 2002b)
A909 Δbac		A909 with <i>bac</i> gene deleted	(Areschoug, Stålhammar-Carlemalm, et al. 2002b)
A909 Δbac + pLZ. <i>bac</i>	pLZ. <i>bac</i>	A909 Δbac expressing surface β protein	(Areschoug, Stålhammar-Carlemalm, et al. 2002b)

Table 3.1. *S. agalactiae* strains used in Chapter 3.

Strain	Vector	Notes	Reference
<i>Lactococcus lactis</i>			
H1186 + pOri23	pOri23	H1186 carrying pOri23	Sriskandan
MG1363+ pOri23. <i>bac</i>	pOri23. <i>bac</i>	MG1363 expressing surface β protein	This study
MG1363+ pOri23. <i>bac</i> $\Delta B75KN$	pOri23. <i>bac</i> $\Delta B75KN$	MG1363 expressing surface $\beta\Delta B75KN$ -CTD protein	This study
MG1363+ pOri23. <i>bac</i> $\Delta B6C$	pOri23. <i>bac</i> $\Delta B6C$	MG1363 expressing surface $\beta\Delta B6C$ protein	This study
MG1363+ pOri23. <i>bac</i> $\Delta B6C\Delta B75KN$	pOri23. <i>bac</i> $\Delta B6C\Delta B75KN$	MG1363 expressing surface $\beta\Delta B6C\Delta B75KN$ -CTD protein	This study

Table 3.2. *L. lactis* strains used in Chapter 3.

Strain	Vector	Notes	Reference
<i>Escherichia coli</i>			
<i>For carriage of template vectors</i>			
TOP10 + pRSET-C	pRSET-C	TOP10 carrying pRSET-C vector	van Sorge et al. 2021)
DH5α + pRSET-C	pRSET-C	DH5α carrying pRSET-C vector	van Sorge et al. 2021)
DH5α + pOri23	pOri23	DH5α carrying pOri23 vector	Sriskandan
<i>For carriage of truncated recombinant His-tag β vectors</i>			
TOP10 + pRSETC.B6N	pRSETC.B6N	TOP10 carrying pRSETC.B6N vector	van Sorge et al. 2021)
TOP10 + pRSETC.IgABR	pRSETC.IgABR	TOP10 carrying pRSETC.IgABR vector	van Sorge et al. 2021)
TOP10 + pRSETC.B6C	pRSETC.B6C	TOP10 carrying pRSETC.B6C vector	van Sorge et al. 2021)
TOP10 + pRSETC.IgI3	pRSETC.IgI3	TOP10 carrying pRSETC.IgI3 vector	van Sorge et al. 2021)
TOP10 + pRSETC.B75KN	pRSETC.B75KN	TOP10 carrying pRSETC.B75KN vector	van Sorge et al. 2021)
<i>For expression of truncated recombinant His-tag β proteins</i>			
Rosetta Gami + pRSETC.B6N	pRSETC.B6N	Rosetta Gami expressing B6N His-tag protein	van Sorge et al. 2021)
Rosetta Gami + pRSETC.IgABR	pRSETC.IgABR	Rosetta Gami expressing IgABR His-tag protein	van Sorge et al. 2021)
Rosetta Gami + pRSETC.B6C	pRSETC.B6C	Rosetta Gami expressing B6C His-tag protein	van Sorge et al. 2021)
Rosetta Gami + pRSETC.IgI3	pRSETC.IgI3	Rosetta Gami expressing IgI3 His-tag protein	van Sorge et al. 2021)

Rosetta Gami + pRSETC.B75KN	pRSETC.B75KN	Rosetta Gami expressing B75KN His-tag protein	van Sorge et al. 2021)
<i>For carriage of B6C His-tag vectors</i>			
DH5 α + pRSET-C.B6C.L317A	pRSET-C.B6C.L317A	DH5 α carrying pRSET-C.B6C.L317A vector	This study
DH5 α + pRSET-C.B6C.Q321A	pRSET-C.B6C.Q321A	DH5 α carrying pRSET-C.B6C.Q321A vector	This study
DH5 α + pRSET-C.B6C.P328A	pRSET-C.B6C.P328A	DH5 α carrying pRSET-C.B6C.P328A vector	This study
DH5 α + pRSET-C.B6C.E332A	pRSET-C.B6C.E332A	DH5 α carrying pRSET-C.B6C.E332A vector	This study
DH5 α + pRSET-C.B6C.K336A	pRSET-C.B6C.K336A	DH5 α carrying pRSET-C.B6C.K336A vector	This study
DH5 α + pRSET-C.B6C.E343A	pRSET-C.B6C.E343A	DH5 α carrying pRSET-C.B6C.E343A vector	This study
DH5 α + pRSET-C.B6C.E349A	pRSET-C.B6C.E349A	DH5 α carrying pRSET-C.B6C.E349A vector	This study
DH5 α + pRSET-C.B6C.H354A	pRSET-C.B6C.H354A	DH5 α carrying pRSET-C.B6C.H354A vector	This study
DH5 α + pRSET-C.B6C.E357A	pRSET-C.B6C.E357A	DH5 α carrying pRSET-C.B6C.E357A vector	This study
DH5 α + pRSET-C.B6C.D362A	pRSET-C.B6C.D362A	DH5 α carrying pRSET-C.B6C.D362A vector	This study
DH5 α + pRSET-C.B6C.K377A	pRSET-C.B6C.K377A	DH5 α carrying pRSET-C.B6C.K377A vector	This study
DH5 α + pRSET-C.B6C.K388A	pRSET-C.B6C.K388A	DH5 α carrying pRSET-C.B6C.K388A vector	This study
DH5 α + pRSET-C.B6C.S396A	pRSET-C.B6C.S396A	DH5 α carrying pRSET-C.B6C.S396A vector	This study
<i>For expression of B6C His-tag proteins</i>			

Rosetta Gami + pRSET-C.B6C.L317A	pRSET-C.B6C.L317A	Rosetta Gami expressing B6C.L317A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.Q321A	pRSET-C.B6C.Q321A	Rosetta Gami expressing B6C.Q321A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.P328A	pRSET-C.B6C.P328A	Rosetta Gami expressing B6C.P328A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.E332A	pRSET-C.B6C.E332A	Rosetta Gami expressing B6C.E332A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.K336A	pRSET-C.B6C.K336A	Rosetta Gami expressing B6C.K336A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.E343A	pRSET-C.B6C.E343A	Rosetta Gami expressing B6C.E343A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.E349A	pRSET-C.B6C.E349A	Rosetta Gami expressing B6C.E349A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.H354A	pRSET-C.B6C.H354A	Rosetta Gami expressing B6C.H354A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.E357A	pRSET-C.B6C.E357A	Rosetta Gami expressing B6C.E357A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.D362A	pRSET-C.B6C.D362A	Rosetta Gami expressing B6C.D362A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.K377A	pRSET-C.B6C.K377A	Rosetta Gami expressing B6C.K377A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.K388A	pRSET-C.B6C.K388A	Rosetta Gami expressing B6C.K388A His-tag protein	This study
Rosetta Gami + pRSET-C.B6C.S396A	pRSET-C.B6C.S396A	Rosetta Gami expressing B6C.S396A His-tag protein	This study
<i>For carriage of B75KN-CTD His-tag vectors</i>			
DH5α + pRSET-C.B75KN-CTD.E198A	pRSET-C.B75KN-CTD.E198A	DH5α carrying pRSET-C.B75KN-CTD.E198A vector	This study

DH5α + pRSET-C.B75KN-CTD.Q201A	pRSET-C.B75KN-CTD.Q201A	DH5α carrying pRSET-C.B75KN-CTD.Q201A vector	This study
DH5α + pRSET-C.B75KN-CTD.K205A	pRSET-C.B75KN-CTD.K205A	DH5α carrying pRSET-C.B75KN-CTD.K205A vector	This study
DH5α + pRSET-C.B75KN-CTD.S211A	pRSET-C.B75KN-CTD.S211A	DH5α carrying pRSET-C.B75KN-CTD.S211A vector	This study
DH5α + pRSET-C.B75KN-CTD.Y213A	pRSET-C.B75KN-CTD.Y213A	DH5α carrying pRSET-C.B75KN-CTD.Y213A vector	This study
DH5α + pRSET-C.B75KN-CTD.S215A	pRSET-C.B75KN-CTD.S215A	DH5α carrying pRSET-C.B75KN-CTD.S215A vector	This study
DH5α + pRSET-C.B75KN-CTD.K216A	pRSET-C.B75KN-CTD.K216A	DH5α carrying pRSET-C.B75KN-CTD.K216A vector	This study
DH5α + pRSET-C.B75KN-CTD.L218A	pRSET-C.B75KN-CTD.L218A	DH5α carrying pRSET-C.B75KN-CTD.L218A vector	This study
DH5α + pRSET-C.B75KN-CTD.D219A	pRSET-C.B75KN-CTD.D219A	DH5α carrying pRSET-C.B75KN-CTD.D219A vector	This study
DH5α + pRSET-C.B75KN-CTD.V221A	pRSET-C.B75KN-CTD.V221A	DH5α carrying pRSET-C.B75KN-CTD.V221A vector	This study
DH5α + pRSET-C.B75KN-CTD.H222A	pRSET-C.B75KN-CTD.H222A	DH5α carrying pRSET-C.B75KN-CTD.H222A vector	This study
DH5α + pRSET-C.B75KN-CTD.H230A	pRSET-C.B75KN-CTD.H230A	DH5α carrying pRSET-C.B75KN-CTD.H230A vector	This study
DH5α + pRSET-C.B75KN-CTD.I233A	pRSET-C.B75KN-CTD.I233A	DH5α carrying pRSET-C.B75KN-CTD.I233A vector	This study
DH5α + pRSET-C.B75KN-CTD.V234A	pRSET-C.B75KN-CTD.V234A	DH5α carrying pRSET-C.B75KN-CTD.V234A vector	This study
DH5α + pRSET-C.B75KN-CTD.E257A	pRSET-C.B75KN-CTD.E257A	DH5α carrying pRSET-C.B75KN-CTD.E257A vector	This study
DH5α + pRSET-C.B75KN-CTD.H265A	pRSET-C.B75KN-CTD.H265A	DH5α carrying pRSET-C.B75KN-CTD.H265A vector	This study

DH5α + pRSET-C.B75KN-CTD.K280A	pRSET-C.B75KN-CTD.K280A	DH5α carrying pRSET-C.B75KN-CTD.K280A vector	This study
DH5α + pRSET-C.B75KN-CTD.E283A	pRSET-C.B75KN-CTD.E283A	DH5α carrying pRSET-C.B75KN-CTD.E283A vector	This study
<i>For expression of B75KN-CTD His-tag proteins</i>			
Rosetta Gami + pRSET-C.B75KN-CTD.E198A	pRSET-C.B75KN-CTD.E198A	Rosetta Gami expressing B75KN-CTD.E198A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.Q201A	pRSET-C.B75KN-CTD.Q201A	Rosetta Gami expressing B75KN-CTD.Q201A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.K205A	pRSET-C.B75KN-CTD.K205A	Rosetta Gami expressing B75KN-CTD.K205A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.S211A	pRSET-C.B75KN-CTD.S211A	Rosetta Gami expressing B75KN-CTD.S211A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.Y213A	pRSET-C.B75KN-CTD.Y213A	Rosetta Gami expressing B75KN-CTD.Y213A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.S215A	pRSET-C.B75KN-CTD.S215A	Rosetta Gami expressing B75KN-CTD.S215A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.K216A	pRSET-C.B75KN-CTD.K216A	Rosetta Gami expressing B75KN-CTD.K216A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.L218A	pRSET-C.B75KN-CTD.L218A	Rosetta Gami expressing B75KN-CTD.L218A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.D219A	pRSET-C.B75KN-CTD.D219A	Rosetta Gami expressing B75KN-CTD.D219A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.V221A	pRSET-C.B75KN-CTD.V221A	Rosetta Gami expressing B75KN-CTD.V221A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.H222A	pRSET-C.B75KN-CTD.H222A	Rosetta Gami expressing B75KN-CTD.H222A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.H230A	pRSET-C.B75KN-CTD.H230A	Rosetta Gami expressing B75KN-CTD.H230A His-tag protein	This study

Rosetta Gami + pRSET-C.B75KN-CTD.I233A	pRSET-C.B75KN-CTD.I233A	Rosetta Gami expressing B75KN-CTD.I233A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.V234A	pRSET-C.B75KN-CTD.V234A	Rosetta Gami expressing B75KN-CTD.V234A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.E257A	pRSET-C.B75KN-CTD.E257A	Rosetta Gami expressing B75KN-CTD.E257A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.H265A	pRSET-C.B75KN-CTD.H265A	Rosetta Gami expressing B75KN-CTD.H265A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.K280A	pRSET-C.B75KN-CTD.K280A	Rosetta Gami expressing B75KN-CTD.K280A His-tag protein	This study
Rosetta Gami + pRSET-C.B75KN-CTD.E283A	pRSET-C.B75KN-CTD.E283A	Rosetta Gami expressing B75KN-CTD.E283A His-tag protein	This study
<i>For carriage of surface-expressed wildtype β protein vector</i>			
DH5a + pOri23. <i>bac</i>	pOri23. <i>bac</i>	DH5a carrying pOri23. <i>bac</i> vector	This study
<i>For carriage of surface-expressed β protein mutants vectors</i>			
DH5a + pOri23. <i>bac</i> Δ E357	pOri23. <i>bac</i> Δ E357	DH5a carrying pOri23. <i>bac</i> Δ E357 vector	This study
DH5a + pOri23. <i>bac</i> Δ E357 Δ L218	pOri23. <i>bac</i> Δ E357 Δ L218	DH5a carrying pOri23. <i>bac</i> Δ E357 Δ L218 vector	This study

Table 3.3. *E. coli* strains used in Chapter 3.

3.2.2 Constructing vectors

Construction of recombinant truncated β His-tag proteins vectors

Vectors (**Table 3.4**) for expression in *E. coli* and purification of rB6N-His, rIgA-His (IgABR), rB6C-His, rIgI3-His (β -IgSF), and rB75KN-His (β 75KN) were previously constructed by van Sorge and colleagues (van Sorge et al. 2021). In brief, a PCR was set up incubating 1 μ L of forward primer (20mM), 1 μ L of reverse primer (20mM), 1 μ L of pRSET-C.B75KN as a template vector (10 μ g/ml), 12.5 μ L Phusion High-Fidelity DNA polymerase (Thermo

Fisher Scientific), and 9.5µL dH₂O water. Standard protocol reaction mixtures were used with thermocycling conditions of 2 min at 98°C, 35 cycles of 15 s at 98°C, 30 s at 62°C, and 3 min at 72°C, followed by 5 min at 72°C. DNA of an amplicon of expected size was purified from a 1% (w/v) agarose gel using GeneJET Gel Extraction Kit (Thermo Fisher Scientific), digested with Sall (ThermoFischer) and NotI (NEB), and ligated using DNA ligase (NEB) into linearized pRSET-C vector following manufacturer protocols. Plasmids generated were transformed into *E. coli* DH5α cells, and transformants were selected for by growth at 37°C on Luria-Bertani (LB) agar supplemented with 100 µg/ml ampicillin. Transformants carrying pRSET-C vectors containing inserts were screened by colony PCR using DreamTaq Green PCR Master Mix 2× (Thermo Fisher Scientific) and primers amplifying across the insertion site of pRSET-C (**Table 3.5**). Colonies that contained inserts were sent for sequencing by Eurofins. B75KN-CTD and B75KN-NTD vectors were constructed by collaborators in the Geisbrecht group (Xu et al. 2022), by amplifying domains from gBlocks.

Vector	Description	Reference
<i>Template vectors for bacterial protein expression</i>		
pRSET-C	<i>E. coli</i> vector for expression of C-terminal His-tag recombinant proteins controlled by T7 promoter, with a His tag sequence downstream of MCS, and ampicillin resistance gene for selection	(Invitrogen)
pOri23	<i>E.coli-L.lactis</i> shuttle vector for surface-protein expression in <i>L. lactis</i> controlled by p23 promoter and containing erythromycin gene for selection	Sriskandan
<i>For expression of recombinant wildtype His-tag β protein</i>		
pRSET-C. <i>bac</i>	pRSET-C carrying <i>bac</i> gene under control of T7 promoter	This study
pLZ. <i>bac</i>	pLZ carrying <i>bac</i> gene under control of <i>lac</i> promoter	(Areschoug, Stålhammar-Carlemalm, et al. 2002b)
<i>For expression of truncated His-tag β protein mutants</i>		
pRSETC.B6N	pRSET-C carrying B6N insert under control of T7 promoter	(van Sorge et al. 2021)

pRSETC.IgABR	pRSET-C carrying IgA insert under control of T7 promoter	(van Sorge et al. 2021)
pRSETC.B6C	pRSET-C carrying B6C insert under control of T7 promoter	(van Sorge et al. 2021)
pRSETC.IgI3	pRSET-C carrying IgI3 insert under control of T7 promoter	(van Sorge et al. 2021)
pRSETC.B75KN	pRSET-C carrying B75KN insert under control of T7 promoter	(van Sorge et al. 2021)
pRSETC.B75KN-CTD	pRSET-C carrying B75KN-CTD insert under control of T7 promoter	(Xu et al. 2022)
pRSETC.B75KN-NTD	pRSET-C carrying B75KN-NTD insert under control of T7 promoter	(Xu et al. 2022)
<i>For expression of B6C His-Tag mutants</i>		
pRSET-C.B6C.L317A	pRSET-C carrying B6C.L317A insert under control of T7 promoter	This study
pRSET-C.B6C.Q321A	pRSET-C carrying B6C.Q321A insert under control of T7 promoter	This study
pRSET-C.B6C.P328A	pRSET-C carrying B6C.P328A insert under control of T7 promoter	This study
pRSET-C.B6C.E332A	pRSET-C carrying B6C.E332A insert under control of T7 promoter	This study
pRSET-C.B6C.K336A	pRSET-C carrying B6C.K336A insert under control of T7 promoter	This study
pRSET-C.B6C.E343A	pRSET-C carrying B6C.E343A insert under control of T7 promoter	This study
pRSET-C.B6C.E349A	pRSET-C carrying B6C.E349A insert under control of T7 promoter	This study
pRSET-C.B6C.H354A	pRSET-C carrying B6C.E357A insert under control of T7 promoter	This study

pRSET-C.B6C.E357A	pRSET-C carrying B6C.E357A insert under control of T7 promoter	This study
pRSET-C.B6C.D362A	pRSET-C carrying B6C.K377A insert under control of T7 promoter	This study
pRSET-C.B6C.K377A	pRSET-C carrying B6C.K377A insert under control of T7 promoter	This study
pRSET-C.B6C.K388A	pRSET-C carrying B6C.K388A insert under control of T7 promoter	This study
pRSET-C.B6C.S396A	pRSET-C carrying B6C.S396A insert under control of T7 promoter	This study
<i>For expression of B75KN-CTD His-tag mutants</i>		
pRSET-C.B75KN-CTD.E198A	pRSET-C carrying B75KN-CTD.E198A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.Q201A	pRSET-C carrying B75KN-CTD.Q201A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.K205A	pRSET-C carrying B75KN-CTD.K205A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.S211A	pRSET-C carrying B75KN-CTD.S211A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.Y213A	pRSET-C carrying B75KN-CTD.Y213A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.S215A	pRSET-C carrying B75KN-CTD.S215A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.K216A	pRSET-C carrying B75KN-CTD.K216A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.L218A	pRSET-C carrying B75KN-CTD.L218A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.D219A	pRSET-C carrying B75KN-CTD.D219A insert under control of T7 promoter	This study

pRSET-C.B75KN-CTD.V221A	pRSET-C carrying B75KN-CTD.V221A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.H222A	pRSET-C carrying B75KN-CTD.H230A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.H230A	pRSET-C carrying B75KN-CTD.H230A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.I233A	pRSET-C carrying B75KN-CTD.I233A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.V234A	pRSET-C carrying B75KN-CTD.V234A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.E257A	pRSET-C carrying B75KN-CTD.E257A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.H265A	pRSET-C carrying B75KN-CTD.H265A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.K280A	pRSET-C carrying B75KN-CTD.K280A insert under control of T7 promoter	This study
pRSET-C.B75KN-CTD.E283A	pRSET-C carrying B75KN-CTD.E283A insert under control of T7 promoter	This study
<i>For surface expression of β proteins in L. lactis</i>		
pOri23. <i>bac</i>	pOri23 carrying <i>bac</i> gene under control of p23 promoter	This study
pOri23. <i>bac</i> Δ B6C	pOri23 carrying <i>bac</i> Δ B6C insert under control of p23 promoter	This study
pOri23. <i>bac</i> Δ B75KN	pOri23 carrying <i>bac</i> Δ B75KN-CTD insert under control of p23 promoter	This study
pOri23. <i>bac</i> Δ E357	pOri23 carrying <i>bac</i> Δ E357 insert under control of p23 promoter	This study
pOri23. <i>bac</i> Δ E357 Δ L218	pOri23 carrying <i>bac</i> Δ E357 Δ L218 insert under control of p23 promoter	This study
<i>For surface expression of LILRB3 in U937 cell lines</i>		

RP172	Dual promoter lentiviral vector	(Zhao et al. 2020)
RP172.LILRB3	RP172 carrying LILRB3 insert under control of human EF1A promoter for surface expression of LILRB3	(Zhao et al. 2020)

Table 3.4. Vectors used in Chapter 3.

Oligonucleotide	Sequence (5' - 3')
<i>To amplify A909 bac gene from bac gBlock</i>	
<i>bac-F</i>	TATGAATGACAATGATGTTGGATCCATGTTTAAATCTAATTATG
<i>bac-R</i>	ATCGATAAGCTTGGCTGCAGGTCGACTCATGATTTTCTTCTTTTCATTGC
<i>For screening pRSET-C for inserts</i>	
T7-F	TAATACGACTCACTATAGGG
T7-R	CTAGTTATTGCTCAGCGGT
<i>To construct truncated pRSET-C.bac vectors</i>	
<i>For site-directed mutagenesis of pRSET-C.B6C vectors (alanine substitution)</i>	
F-L317A	AAT CAG TTT <u>GCT</u> GAG AAC GCG CAA AAA TTG AAA GAA
R-L317A	CGC GTT CTC <u>AGC</u> AAA CTG ATT CTT TAC CTG CGT CGC
F-Q321A	GAG AAC GCG <u>GCT</u> AAA TTG AAA GAA ATT CAA CCC TTA
R-Q321A	TTT CAA TTT <u>AGC</u> CGC GTT CTC CAG AAA CTG ATT CTT
F-P328A	GAA ATT CAA <u>GCT</u> TTA ATA AAG GAA ACT AAT GTA AAA
R-P328A	CTT TAT TAA <u>AGC</u> TTG AAT TTC TTT CAA TTT TTG CGC
F-E332A	TTA ATA AAG <u>GCT</u> ACT AAT GTA AAA CTG TAC AAA GCC
R-E332A	TAC ATT AGT <u>AGC</u> CTT TAT TAA GGG TTG AAT TTC TTT
F-K336A	ACT AAT GTA <u>GCT</u> CTG TAC AAA GCC ATG TCC GAA AGC
R-K336A	TTT GTA CAG <u>AGC</u> TAC ATT AGT TTC CTT TAT TAA GGG

F-E343A	GCC ATG TCC <u>GCT</u> AGC CTG GAG CAA GTC GAG AAA GAA
R-E343A	CTC CAG GCT <u>AGC</u> GGA CAT GGC TTT GTA CAG TTT TAC
F-E349A	GAG CAA GTC <u>GCT</u> AAA GAA TTA AAG CAT AAC TCA GAA
R-E349A	TAA TTC TTT <u>AGC</u> GAC TTG CTC CAG GCT TTC GGA CAT
F-H354A	GAA TTA AAG <u>GCT</u> AAC TCA GAA GCA AAC TTG GAA GAT
R-H354A	TTC TGA GTT <u>AGC</u> CTT TAA TTC TTT CTC GAC TTG CTC
F-E357A	CAT AAC TCA <u>GCT</u> GCA AAC TTG GAA GAT TTA GTA GCC
R-E357A	CAA GTT TGC <u>AGC</u> TGA GTT ATG CTT TAA TTC TTT CTC
F-D362A	AAC TTG GAA <u>GCT</u> TTA GTA GCC AAG TCA AAG GAG ATA
R-D362A	GGC TAC TAA <u>AGC</u> TTC CAA GTT TGC TTC TGA GTT ATG
F-K377A	TAT GAA GGG <u>GCT</u> TTG AAT CAG TCA AAA AAT CTG CCT
R-K377A	CTG ATT CAA <u>AGC</u> CCC TTC ATA TTC CCG CAC TAT CTC
F-K388A	CCT GAA TTA <u>GCT</u> CAA TTA GAA GAG GAG GCG CAT AGC
R-K388A	TTC TAA TTG <u>AGC</u> TAA TTC AGG CAG ATT TTT TGA CTG
F-S396A	GAG GCG CAT <u>GCT</u> AAA CTG AAG CAG GTC GTG GAA GAC
R-S396A	CTT CAG TTT <u>AGC</u> ATG CGC CTC CTC TTC TAA TTG TTT
<i>For site-directed mutagenesis of pRSET-C.B75KN-CTD vectors (alanine substitution)</i>	
E198A-F	<u>GAA AAC TCT GCC ATG GAC CAG GCT AAA GAG AAG</u>
E198A-R	<u>CTG GTC CAT GGC AGA GTT TTC TGG AAT TGG TGC</u>
Q201A-F	GAA ATG GAC <u>GCC</u> GCT AAA GAG AAG GCG AAG ATC GCG
Q201A-R	CTC TTT AGC <u>GGC</u> GTC CAT TTC AGA GTT TTC TGG AAT
K205A-F	GAG AAG GCG <u>GCC</u> ATC GCG GTC TCG AAG TAT ATG AGC
K205A-R	GAC CGC GAT <u>GGC</u> CGC CTT CTC TTT AGC CTG GTC CAT
S211A-F	ATC GCG GTC <u>GCC</u> AAG TAT ATG AGC AAA GTG TTA GAT
S211A-R	CAT ATA CTT <u>GCC</u> GAC CGC GAT CTT CGC CTT CTC TTT
Y213A-F	GTC TCG AAG <u>GCC</u> ATG AGC AAA GTG TTA GAT GGA GTA

Y213A-R	TTT GCT CAT <u>GGC</u> CTT CGA GAC CGC GAT CTT CGC CTT
S215A-F	AAG TAT ATG <u>GCC</u> AAA GTG TTA GAT GGA GTA CAT CAA
S215A-R	TAA CAC TTT <u>GGC</u> CAT ATA CTT CGA GAC CGC GAT CTT
K216A-F	TAT ATG AGC <u>GCC</u> GTG TTA GAT GGA GTA CAT CAA CAT
K216A-R	TAT ATG AGC <u>GCC</u> GTG TTA GAT GGA GTA CAT CAA CAT
L218A-F	AGC AAA GTG <u>GCC</u> GAT GGA GTA CAT CAA CAT TTA CAG
L218A-R	TAC TCC ATC <u>GGC</u> CAC TTT GCT CAT ATA CTT CGA GAC
D219A-F	AAA GTG TTA <u>GCC</u> GGA GTA CAT CAA CAT TTA CAG AAA
D219A-R	ATG TAC TCC <u>GGC</u> TAA CAC TTT GCT CAT ATA CTT CGA
V221A-F	TTA GAT GGA <u>GCC</u> CAT CAA CAT TTA CAG AAA AAA AAC
V221A-R	ATG TTG ATG <u>GGC</u> TCC ATC TAA CAC TTT GCT CAT ATA
H230A-F	AAA AAA AAC <u>GCC</u> TCG AAG ATT GTT CTG TTT AAG
H230A-R	AAT CTT CGA <u>GGC</u> GTT TTT TTT CTG TAA ATG TTG ATG
I233A-F	CAT TCG AAG <u>GCC</u> GTT GAT CTG TTT AAG GAA CTT
I233A-R	CAG ATC AAC <u>GGC</u> CTT CGA ATG GTT TTT TTT CTG TAA
V234A-F	TCG AAG ATT <u>GCC</u> GAT CTG TTT AAG GAA CTT GAG GCG
V234A-R	AAA CAG ATC <u>GGC</u> AATCTTCGAATGGTTTTTTTCTG
E257A-F	GCC AAA ACG <u>GCC</u> GTG GAA ATT GAC AAT TTA GTA CAT
E257A-R	AAT TTC CAC <u>GGC</u> CGTTTTGGCGTTATCAATGTCGAA
H265A-F	AAT TTA GTA <u>GCC</u> GAC GCA TTC TCA AAG ATG AAT GCC
H265A-R	GAA TGC GTC <u>GGC</u> TACTAAATTGTCAATTTCCACTTC
K280A-F	AAG TTT CAA <u>GCC</u> GGA TTA GAG ACT AAT CTG CCT AGT
K280A-R	CTC TAA TCC <u>GGC</u> TTGAACTTAGCTACGGTGGCATT
E228A-F	<u>AAA GGA TTA GCC ACT AAT CTG CCT AGT ACG GGG</u>
E228A-R	<u>CAG ATT AGT GGC TAA TCC TTT TTG AAA CTT AGC</u>
For site-directed mutagenesis of pOri23.bac vectors (alanine substitution)	

E357A-F	AGT AAG GTT <u>GCC</u> GAT GGA GTT CAT CAA CAT CTG
E357A-R	ATG AAC TCC <u>ATC</u> GGC AAC CTT ACT CAT ATA CTT
L218A-F	CAT AAT TCG <u>GCC</u> GCT AAT TTA GAA GAT TTG
L218A-R	TAA ATT AGC <u>GGC</u> CGA ATT ATG TTT TAA TTC

Table 3.5. Primers used in Chapter 3.

Site-directed mutagenesis by inverse PCR of pRSET-C.B6C, pRSET-C.B75KN and pOri23.bac vectors to introduce single alanine substitutions

To construct vectors that expressed B6C and B75KN with a C-terminal His tagged containing single alanine substitutions at specific residues, site-directed mutagenesis by inverse PCR using pRSET-C.B6C and pRSET-C.B75KN as template vectors was performed (**Table 3.4**). Primers were designed that amplified pRSET-C.B6C and pRSET-C.B75KN vectors introducing a single alanine substitution (**Table 3.5**). Forward primers were designed to have 9 nucleotides complementary to the sequence upstream of the site of alanine substitution, a codon encoding alanine (GCT/GCC), and 24 nucleotides complementary to the downstream of the site of alanine substitution. Reverse primers were designed with 9 nucleotides reverse complemented to downstream of the alanine substitution, a reverse complement codon for alanine (AGC/GGC), and 24 nucleotides reverse complemented to upstream of the alanine substitution. For each vector to construct, 1 µl of forward primer (20 mM), 1 µl of reverse primer (20 mM), 1 µL of template vector (5 ng/ul), 12.5 µl Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), and 9.5 µl dH₂O water were mixed. Thermocycling was performed under the following conditions: 2 mins at 98°C, 25 cycles of 15 s at 98°C, 30 s at 62°C, and 5 mins at 72°C, followed by a final cycle of 5 mins at 72°C. A small volume (4 ul) of the PCR product was run on a 1% agarose gel to confirm successful amplification. The PCR product was digested with using *DpnI* (Promega) following manufacturer's protocols to degrade non-amplified template plasmid. 5 µl of the resulting *DpnI* digested amplicon was transformed into *E. coli* DH5α and transformed colonies were selected by growth on LB agar plates supplemented with 100 µg/ml ampicillin. pRSET-C plasmids from transformants were prepared following from *E. coli* cultures in LB media supplemented with 100 µg/ml ampicillin using the GeneJet Plasmid Miniprep kit (ThermoFisher), and sent for sequencing to confirm the sequence of the inserts.

Construction of heterologous expression vectors

pOri23 *E. coli*–*L. lactis* shuttle vectors were constructed and used for heterologous expression of β protein in *L. lactis* (Que et al. 2000). PCR was used to amplify the *bac* gene from GBS strain A909 using the specific primers (**Table 3.5**) and Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). For the reaction mixture, 1 µl

of forward primer (20 mM), 1 µl of reverse primer (20 mM), 1 µl of lysed bacterial colony previously resuspended in 20 µl dH₂O, 12.5 µl Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), and 9.5 µl dH₂O water were mixed. Standard protocol reaction mixtures were used with thermocycling conditions of 2 mins at 98°C, 35 cycles of 15 s at 98°C, 30 s at 62°C, and 3 mins at 72°C, followed by 5 mins at 72°C. DNA of an amplicon of expected size (3.4 kb) was purified from a 1% (w/v) agarose gel using GeneJET Gel Extraction Kit (Thermo Fisher Scientific), digested with *Bam*HI and *Sal*I (New England Biolabs), and ligated into *Bam*HI/*Sal*I linearized pOri23 vector following manufacturer's protocols. Plasmids generated were transformed into *E. coli* DH5α cells, and transformants were selected for by growth at 37°C on Luria-Bertani (LB) agar supplemented with 500 µg/ml erythromycin. Transformants containing pOri23 vectors with the *bac* gene (pOri23.*bac*) were screened by colony PCR using DreamTaq Green PCR Master Mix 2× (Thermo Fisher Scientific) and primers amplifying across the insertion site of pOri23.

The wild-type pOri23.*bac* vector was used as the template for inverse PCR to construct: (1) pOri23.*bac*ΔB75KN, a vector encoding β protein with an in-frame deletion of the B75KN domain (residues 504–788), using primers specific to the IgI3 (5'-GATCCGCGGCCGCTTGTTCCTCTCTTTTGC-3') and XPZ (5'-GATCGCGGCCGCGACGCCAGAACTCCAGATACACCG-3') domains and containing NotI restriction sites. For the reaction mixture, 1 µl of forward primer (20 mM), 1 µl of reverse primer (20 mM), 1 µl of template vector (diluted to 5 ng/ul), 12.5 µl Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), and 9.5 µl dH₂O water were mixed. Thermocycling was performed under the following conditions: 2 mins at 98°C, 25 cycles of 15 s at 98°C, 30 s at 62°C, and 5 mins at 72°C, followed by a final cycle of 5 mins at 72°C. A small volume (4 µl) of the PCR product was run on a 1% agarose gel to confirm successful amplification. Subsequently, the PCR product was digested with *Dpn*I (Promega), followed by *Not*I digestion, and then ligated into *Not*I linearized pOri23 vector following manufacturer's protocols. The vectors were then transformed into *E. coli* DH5α cells. Transformants carrying pOri23.*bac* vectors were selected by growth on LB + 500 µg/ml erythromycin agar plates and individual colonies were screened by colony PCR using DreamTaq Green PCR Master Mix 2× (Thermo Fisher Scientific). For the reaction mixture, 1 µl of forward primer (20 mM), 1 µl of reverse primer (20 mM), 1 µl of lysed bacterial colony previously resuspended in 20 µl dH₂O, 12.5 µl DreamTaq Green polymerase (Thermo Fisher Scientific), and 9.5 µl dH₂O water were mixed. PCR was set to 95°C for 5 mins, 35 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 60 s, followed by 72°C for 10 mins. PCR products were separated on a 1.25% agarose gel by gel electrophoresis to assess the size of the vector insert.

Producing chemically competent E. coli

E. coli were prepared as described in Chapter 2.

Transforming E. coli strains for expression of B6C and B75KN

E. coli was utilised to express recombinant B6C and B75KN proteins. Chemically competent DH5- α or TOP10 *E. coli* strains were transformed to overexpress pRSET-C.B6C and pRSET-C.B75KN constructed vectors and chemically competent RG were transformed to overexpress proteins (**Table 3.4**). *E. coli* was heat-shocked to induce uptake of vector. 50 μ L of DH5- α or RG were incubated with 50-100 ng pRSET-C or pOri23 vector for 20 mins on ice, 1 min 42°C, 2 mins on ice, and finally 120 mins at 37 °C. Cultures were spread and incubated at 37°C overnight on LB + 100 μ g/ml ampicillin or 500 μ g/ml erythromycin agar plates for pRSET-C or pOri23 transformants, respectively. Transformants containing pOri23 or pRSET-C vectors were screened by colony PCR using DreamTaq Green PCR Master Mix 2 $\times$ (Thermo Fisher Scientific) and primers amplifying across the insertion site of pOri23 or pRSET-C.

3.2.3 Expression of recombinant bacterial proteins

Expression and purification of all proteins was performed as previously described (van Sorge et al. 2021). To express high amounts of protein, 300 ml of RG *E. coli* carrying pRSET-C vectors (**Table 3.3**) were cultured in LB broth at 37°C with shaking until an absorbance OD₆₀₀ = 0.5-0.7. Cultures were then supplemented with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and cultured for a further 4 hours at 37°C with shaking. *E. coli* was harvested by centrifugation at 3,273 g for 30 mins, and the *E. coli* pellets were stored overnight at -80°C. *E. coli* was lysed by resuspending pellets in 10 ml native binding buffer (20 mM Na₂HPO₄, 0.5 M NaCl, pH7.8), pooling two resuspensions, supplementing with 200 μ g/ml lysozyme (Sigma) and 1 mM phenylmethylsulphonyl fluoride (PMSF, ThermoFisher), and incubating on ice for 25 mins. *E. coli* samples were further lysed by sonication for 10 seconds at amplitude 50 (ThermoFisher), frozen at -80°C, and thawed in 37°C water bath for 3 cycles. Finally, lysates were filtered by filter units (StarLabs).

rB75KN-His and rB6C-His proteins were purified from filtered lysate samples by affinity chromatography (ÄKTA pure; GE Healthcare Life Sciences) using 5 ml His-Trap Nickel columns (Cytiva, Life Sciences). Prior to use, Nickel columns were washed by passing 25 ml of native binding buffer (20 mM Na₂HPO₄, 0.5 M NaCl, pH 7.8) over the His-Trap column using a peristaltic pump. To load the His-Trap columns with supernatant, samples were passed over the His-Trap Nickel column, followed by 25 ml of native binding buffer, using a peristaltic pump. Proteins were eluted from the His-Trap Nickel column using an ÄKTA pure by passing a gradient of imidazole (10-500 mM) in native binding buffer over the His-Trap column and collecting 1.5 ml elution fractions. As the ÄKTA pure collects and plots data on absorbance of elutions over time (UNICORN system control software), the absorbance curve was used to predict the elution fractions that contained rB75KN-His and rB6C-His. For fractions selected based on absorbance, 50 μ L of the elution sample was mixed with 50 μ L of 2 x sample buffer, boiled at 95°C for 5 mins, and tested for protein expression by SDS-PAGE analysis, as previously described. Elution samples containing a detectable concentration of protein on a stained SDS-PAGE gel were pooled and dialysed in 1X PBS at 4°C overnight in 12 kDa dialysing tubes (SLS). Protein samples were concentrated by centrifugation at 3,273 g using 10 kDa filter concentrating units (Amicon) to volumes of 1-5 ml and samples were stored at -20°C.

3.2.4 Expression of recombinant LILR proteins

Vectors for expression of rLILRB1-His, rLILRB3-His, rLILRB2-Fc, and rLILRB3-Fc proteins were previously constructed, as described by (Zhao et al. 2020). All rLILR-His and rLILR-Fc proteins were previously expressed and available for this study. In brief, 50 µg of pcDNA3.4 vectors was resuspended in 5ml Opti-MEM (1x) (Life Technologies, USA) containing 50 ng/ml polyethylenimine (PEI MAX, Polysciences, USA) and incubated for 20 minutes at room temperature. Next, the vector-PEI-Opti-MEM solution was added to 50 ml of EXPI293 cells cultured to 2×10^6 cells/ml in EXPI293 expression media (Life Technologies, USA), and incubated for 4 days at 37°C with 5% CO₂. After 3 days, the supernatant of EXPI293 cell culture was obtained by centrifugation of cells for 10 mins at 675 g. 50 ml of EXPI293 supernatant was pipetted into 12,000-14,000 Dalton Spectra/Pore dialysis membrane (Spectrum laboratories, USA) and dialysed in 5 L of 50 mM Tris/300 mM NaCl pH8. LILR-His proteins were purified by affinity chromatography (ÄKTA pure; GE Healthcare Life Sciences) using 5 ml His-Trap Nickel columns (GE healthcare, Sweden) and eluted with gradient imidazole (10-500 mM) in 50 mM Tris/ 300 mM NaCl pH8. rLILR-Fc proteins were purified using 5 ml protein G columns (GE healthcare, Sweden) and eluted with 0.1 M Glycine-HCL pH 2.7. Protein purity was tested by running samples on SDS-PAGE and samples were dialysed again.

3.2.5 Construction of *L. lactis* heterologous expression system

Producing chemically competent L. lactis and transformation

Chemically competent *L. lactis* and transformation were performed as described in Chapter 2.

3.2.6 Culture of LILR-expressing human cell lines

The LILRB3-expressing U937 cell line was previously reported (Zhao et al. 2020). U937 cell lines were cultured in RPMI 1640 +10% FCS +1 00µg/ml penicillin and 100µg/ml streptomycin at 37°C with 5% CO₂. The LILRB3CD3ζ 2B4 NFAT-GFP T cell reporter cell line was previously reported (Zhao et al. 2020). 2B4 NFAT-GFP T cell reporter cell line was cultured in RPMI 1640 +10% FCS +1 00µg/ml penicillin and 100µg/ml streptomycin at 37°C with 5% CO₂.

Cell Line	Description	Reference
U937	U937 wildtype	Sigma-Aldrich
U937.LILRB3	LIRB3-expressing U937 cell line	McCarthy
2B4T	2B4T wildtype	McCarthy
2B4T.LILRB3	LILRB3-expressing 2B4T cell line	(Zhao et al. 2020)

Table 3.6. Cell lines used in Chapter 3.

3.2.7 Biochemical assays

Testing proteins for purity by SDS-PAGE

Recombinant β protein purification was measured by SDS-PAGE analysis. 50 μ L of 100 μ g/ml of rB6N, rIgABR, rB6C rIgI3, or rB75KN were incubated with 2X Sample Buffer. Samples were separated on a 12.5% SDS-PAGE gel, washed in double-distilled H₂O, stained with Biosafe Coomassie Blue stain (Bio-Rad Laboratories), and destained with double-distilled H₂O.

Limiting proteolytic digestion of B75KN and mass spectrometry characterisation

Experiments were performed by Geisbrecht lab (Xu et al. 2022).

Surface plasmon resonance analysis of truncated β protein interactions with LILRB3

Experiments were performed by Geisbrecht lab.

Binding of LILRB3-Fc to truncated β protein, B6C-His or B75KN-His coated Dynabeads

The interaction of rLILRB3-Fc with Dynabeads coated with rB75KN-His or rB6C-His proteins was measured by flow cytometry. To prepare rB75KN-His and rB6C-His protein coated Dynabeads, His-tag Dynabeads (Invitrogen; 0.4 μ L per sample) were washed once in wash buffer (1X PBS containing 0.5% (w/v) BSA (Sigma Aldrich) + 10 mM imidazole (Sigma Aldrich)). Dynabead washes were performed by addition of 100 μ L wash buffer, transference to a magnet for 2 mins, aspiration of wash buffer, and resuspension in 100 μ L wash buffer. Dynabeads were coated in rB75KN-His or rB6C-His proteins by incubation with 160 ng of rB75KN-His or rB6C-His (diluted to 100 μ g/ml in 1X PBS + 0.5% BSA + 10 mM imidazole) per sample for 30 mins on ice. His-tag Dynabeads were then washed twice in wash buffer to remove rB75KN-His or rB6C-His proteins.

To measure the rLILRB3-Fc interaction with rB75KN-His or rB6C-His coated Dynabeads, 30 μ L of a 10 μ g/mL solution of rLILRB3-Fc diluted in wash buffer was incubated with rB75KN-His or rB6C-His coated Dynabeads, and incubated on ice for 1 hour at 37°C. Subsequently, the Dynabeads were washed 3 times in wash buffer and stained with goat anti-human-IgG-FITC (1:1,000; Southern Biotech) for 30 mins on ice. Dynabeads were then washed 3 times in wash buffer and resuspended in 30 μ L of the same buffer. Fluorescence was measured by flow cytometry FACS Calibur (Becton Dickinson) and analysed by FlowJo.

3.2.8 Bacterial cell culture assays

*Measuring *L. lactis* β expression by SDS-PAGE*

Total β protein expression was assessed by SDS-PAGE analysis. 1 ml of *L. lactis* overnight cultures were pelleted by centrifugation at 2,250 *g* for 5 mins and resuspended in 100 μ L sample buffer. Bacterial resuspensions were lysed by heating to 95°C for 10 mins then separated on a 12.5% SDS-PAGE gel. The gel was then washed in double-distilled H₂O, stained with Biosafe Coomassie Blue stain (Bio-Rad Laboratories), and destained with double-distilled H₂O.

*Measuring *S. agalactiae* and *L. lactis* β protein expression by flow cytometry*

Bacterial surface expression of β protein was measured by flow cytometry. Rabbit anti- β serum was produced by subcutaneous injection into rabbit of β protein emulsified in Complete Freund's Adjuvant, and collecting serum after 14 days (Lindahl *et al.*, 1990). Mid-logarithmic phase bacteria were incubated for 30 min on ice with either 0.1% (v/v) rabbit anti- β serum or normal rabbit serum (Lindahl *et al.* 1990). After washing in PBS plus 0.5% (w/v) BSA (by centrifugation for 5 mins at 2,250 *g*), bacteria were incubated for 30 min on ice with a 1:1,000

dilution of FITC-conjugated goat anti-rabbit IgG (Life Technologies). After washing in PBS plus 0.5% (w/v) BSA (centrifugation for 5 mins at 2,250 g), bacteria were fixed in 1% (w/v) paraformaldehyde (PFA). Fluorescence of bacteria was measured by flow cytometry analysis (FACS Calibur).

Measuring LILRB3-Fc/His interaction with S. agalactiae and L. lactis by flow cytometry

The LILRB3 binding phenotype of *S. agalactiae* and *L. lactis* strains was measured by flow cytometry using rLILRB3-His (and control proteins) or rLILRB3-Fc (and control proteins). Overnight *S. agalactiae* and *L. lactis* cultures were subcultured 1:10 in TH or GM17, respectively, and grown to mid-logarithmic growth phase $\sim OD=0.4$. 30 μ L of bacterial cultures were incubated with either 10 μ g/ml LILRB3-Fc or control proteins or PBS for 30 min on ice. After washing in PBS plus 0.5% (w/v) BSA (by centrifugation for 5 mins at 2,250 g), bacteria were stained for 30 min on ice with 1:1,000 goat anti-human.IgG.FITC (Southern Biotech). After washing in PBS plus 0.5% (w/v) BSA (by centrifugation for 5 mins at 2,250 g), bacteria were fixed in 1% (v/v) PFA. Fluorescence of bacteria was measured by flow cytometry analysis (FACS Calibur).

3.2.9 Human cell culture assays

Ethical approval and consent for use of human blood

Ethical approval for draw and use of human blood was obtained from the Regional Ethics Committee and Imperial College Healthcare NHS Trust Tissue Bank (Regional Ethics Committee approval no. 17/WA/0161, Imperial College Healthcare Tissue Bank Human Tissue Authority license no. 12275, and Imperial College Research Ethics Committee no. 19IC5166).

2B4T.LILRB3CD3 ζ cell crosslinking assays

2B4 NFAT-GFP T cell reporter cells lines were used to assess LILRB3 crosslinking (Zhao et al. 2020). *S. agalactiae* isogenic A909 strains - A909, Δbac , Δbac + pLZ.*bac*- were subcultured to an absorbance of $OD_{600} = 0.8$. Next, 1 ml of culture was pelleted (5 mins, 2,250 g) and re-suspended in 333 μ L of RPMI 1640 containing 10% FCS. 10 μ L of prepared bacteria (multiplicity of infection (MOI) 200) were added to wells of a 96-well tissue culture plate that had previously been seeded with 200 μ L of 2B4T cells at 2.5×10^5 cells/ml in RPMI 1640. The 2B4 NFAT-GFP T cells were cultured at 37°C with 5% CO₂ for 24 hours. After incubation, 2B4T cells were washed with PBS to remove unadhered *S. agalactiae*, resuspended in 100 μ L PBS and fluorescence of the cells was measured by flow cytometry (FACS Calibur).

Adhesion of GBS to LILRB3 U937 cells

U937 and LILRB3.U937 cells were resuspended to 2×10^6 cell/ml in PBS + 0.1% BSA (Serva, Germany) and 45 μ l volume was seeded to 96-well plates. FITC-labelled isogenic A909 strains were resuspended in PBS + 0.1% BSA (Serva, Germany) to a concentration 2×10^8 CFU/ml. U937 cells were inoculated with FITC-labelled bacteria at an MOI of 10 and incubated for 30 minutes at 4°C. Non-adhered bacteria were washed away by addition of 100 μ l PBS, centrifugation at 675 *g* for 7 minutes at 4°C and removal of the supernatant. The cell pellet was resuspended in 10% PFA. Fluorescence of U937 cells was measured by FACS Verse flow cytometry (BD, USA).

Neutrophil phagocytosis assays

Resistance of *L. lactis* strains to phagocytosis by neutrophils was measured by flow cytometry. Neutrophils were isolated from human blood and the assay was performed as previously described (Zhao et al. 2020). FITC-labelled *L. lactis* strains were generated by incubating stationary phase bacteria with 1 mg/ml FITC (Sigma-Aldrich) for 30 min. To opsonise bacteria with complement, samples were incubated in 3% human serum for 30 mins at room temperature. Human serum was prepared by centrifugation at 1000 *g* of freshly drawn human blood in vacutainer tubes (Thermofisher) and collecting supernatant. Bacteria were washed five times in PBS (centrifuge 2,250 *g* for 5 mins) and incubated in rLILRB3-His or PBS for 30 mins. Neutrophils were incubated with FITC-labelled complement-opsonised *L. lactis* at a MOI of 10 at 37°C for 30 mins with shaking, washed with PBS by addition of 100 μ l PBS and centrifuged at 675 *g* for 7 minutes at 4°C. The cell pellet was fixed in 1% (w/v) PFA. Extracellular bacteria were quenched by addition of 250 μ g/ml trypan blue dissolved in ice-cold 0.1 M citrate buffer (pH 4). Neutrophil fluorescence was then measured by flow cytometry. The results of each assay were expressed as the geometric mean normalized to a control group.

3.2.10 Generating predicted β protein molecular structures

Predicted structures for the β protein and β protein domains - B6C and B75KN-CTD - were generated by alphafold (Varadi et al. 2022; Jumper et al. 2021). Cartoon and ribbon structures with selected residues highlighted were imaged by PyMol (The PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC).

3.3 Results

3.3.1 *S. agalactiae* β protein is a LILRB3 ligand

S. agalactiae can be subdivided into 10 serotypes (Ia, Ib, II-IX) based on the immunogenicity of the polysaccharide capsule (Carlin, Chang, et al. 2009), with certain serotypes associated with more virulent strains that cause invasive disease (Yu et al. 2018). The hypothesis was that the interaction of *S. agalactiae* with LILRB3 was serotype dependent. To test this, a screen of virulent strains belonging to each of the common invasive-disease-associated serotypes Ia, Ib, II, III and V for binding to recombinant LILRB3-Fc was performed (**Fig 3.1A**). The purified rLILRB3-Fc was tested for purity by SDS-PAGE (**Supp. 2.1**). *S. agalactiae* strains were incubated with rLILRB3-Fc and binding was measured by flow cytometry (**Fig 3.1A**). rLILRB3-Fc bound to A909, BS22, H36B, BS29, SBL3006, SB35 and NEM316 strains, but not to BS39, 515, 18RS21, BM110, COHI, and NCTC-10-84. Interestingly, LILRB3 binding to the surface of *S. agalactiae* was not serotype dependent; for example, rLILRB3-Fc bound to A909 of serotype Ia, but not to BS39 and 515 of serotype Ia. Similarly, rLILRB3-Fc bound BS29 but not 18RS21 of serotype II. Thus, LILRB3 does not bind the capsular polysaccharide and must be interacting with another surface ligand.

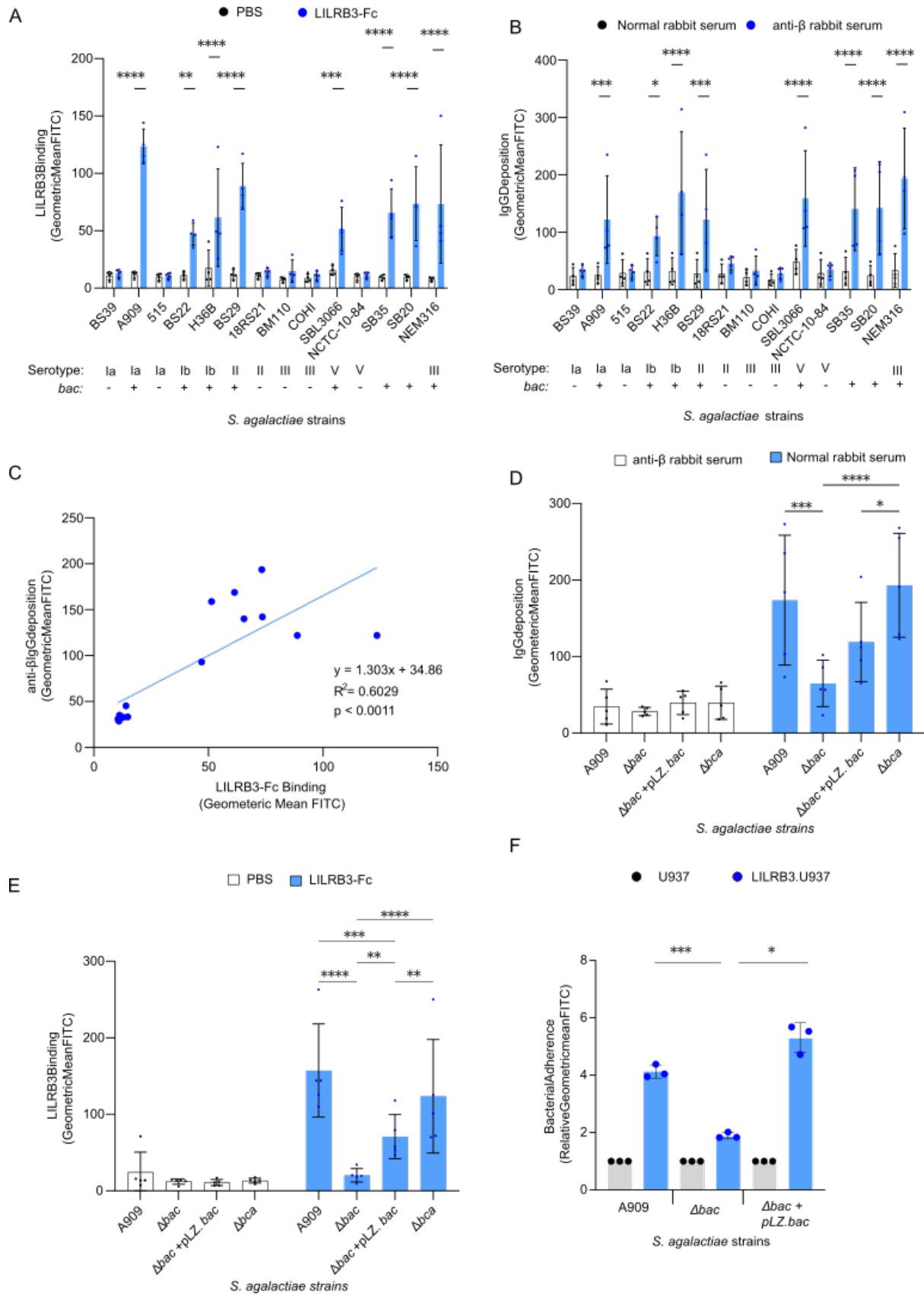


Figure 3.1. *S. agalactiae* binds to rLILRB3-Fc via the β protein. (A) Binding of recombinant LILRB3 (rLILRB3-Fc) to the surface of *S. agalactiae* strains was measured by flow cytometry. Stationary phase *S. agalactiae* strains were incubated with rLILRB3-Fc (10 μ g/mL) or PBS for 30 mins, washed in PBS, stained with goat anti-human-Fc-FITC mAb (1:1,000) for 30 mins. Samples were then washed in PBS and fixed in 1% PFA. *S. agalactiae* serotype and carriage of *bac* gene are represented along the bottom of graph. (B) Surface expression of β protein of *S. agalactiae* was detected by incubation in 0.1% (v/v) anti- β or non-immune rabbit serum flow cytometry analysis. (C) Simple linear regression of correlation of β expression (values in B) and LILRB3 binding (values in A) for *S. agalactiae* strains. A two-tailed p test, and R^2 and two-tailed p value were calculated. (D-E) β surface expression and rLILRB3-Fc binding of A909 strain wildtype, Δbac , Δbac + pLZ.*bac*, and Δbca were measured using the same method as (A) and (B), respectively. (F) Adherence of FITC-labelled isogenic *S. agalactiae* strains to LILRB3-expressing U937 cell lines, measure by flow cytometry. The relative increase in adherence between U937 and LILRB3-expressing U937 was assessed between bacterial strains for statistically significant differences. All experiments were performed in at least three independent experiments with biological replicates shown for each data point, with the mean and SD shown. Two-way ANOVA was performed to assess levels of statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$).

Having excluded the capsular polysaccharide as the LILRB3 ligand, the next hypothesis was that the *S. agalactiae* strains exhibiting LILRB3-binding express a surface protein that is absent in those strains that show no LILRB3 binding. The McCarthy lab previously performed a genome comparison of CEACAM1 binders and non-binders to identify the β protein as the surface GBS protein that binds CEACAM1. Therefore, the same approach was used for LILRB3 binders. Notably, the LILRB3 binding strains matched the CEACAM1 binding strains. In other words, the *bac* gene that encodes the surface β protein, was identified as being carried by all strains that bound LILRB3 but absent in those that did not (Fig 3.1A, bottom panel). The β protein is a large ~130kDa protein expressed on the *S. agalactiae* cell surface. It, together with α protein that is encoded by the *bca* gene, composes the C protein complex that is common among *S. agalactiae* strains (Jerlström, Chhatwal, and Timrnis 1991). Importantly, the β protein is a virulence factor with isogenic β -protein knockout studies demonstrating that β protein inhibits phagocytic uptake of *S. agalactiae* and enhances resistance to whole blood killing (Carlin et al. 2009). Given this information, the hypothesis was that β protein was the LILRB3 ligand expressed by *S. agalactiae*.

To confirm that carriage of the *bac* gene was necessary for LILRB3 interaction, 3 additional strains were tested that were known to contain the *bac* gene - SB35sed, SB20, and NEM316 - and indeed these strains also exhibited LILRB3-binding phenotype (Fig 3.1A). To assess whether surface β protein expression correlated with LILRB3 binding, the surface β expression by *S. agalactiae* strains was tested using anti- β rabbit serum and flow cytometry (Fig 3.1B). rLILRB3-Fc binding to *S. agalactiae* was strongly correlated to β protein expression with R^2

= 0.6029 and $p < 0.0011$ (**Fig 3.1C**). Therefore, the expression of β protein is correlated to LILRB3 binding to *S. agalactiae*.

To determine whether β protein expression was necessary for LILRB3 binding *S. agalactiae* isogenic A909 mutant strains - Δbac , $\Delta bac + pLZ.bac$, and Δbca - were tested for LILRB3 binding. First, β expression was measured using anti- β serum and flow cytometry (**Fig 3.1D**). As expected, all *bac* carrying strains (A909, $\Delta bac + pLZ.bac$, Δbca) exhibited high levels of surface β protein expression whilst the Δbac mutant strain showed no β surface expression (**Fig 3.1D**). Of note, unlike A909 and the Δbca mutant, the complemented strain ($\Delta bac + pLZ.bac$) did not exhibit significantly more IgG deposition than on the Δbac . rLILRB3-Fc binding to isogenic A909 strains was then measured by flow cytometry (**Fig 3.1E**). rLILRB3-Fc bound to A909 but not to Δbac , showing that deletion of the *bac* gene abrogates rLILRB3-Fc binding (**Fig 3.1E**). Furthermore, rLILRB3-Fc binding was recapitulated in the complemented strain $\Delta bac + pLZ.bac$ strain showing that loss of the *bac* gene results in loss of rLILRB3-Fc interaction (**Fig 3.1E**). The levels of LILRB3 binding were not completely restored in the complemented strain which may be due to the lower levels of surface β expression. Deletion of the *bca* gene in A909, encoding α protein, showed no abrogation of rLILRB3-Fc binding, demonstrating that the loss of LILRB3 binding was not due to overall reduction in C protein expression (**Fig 3.1E**).

Finally, the β -LILRB3 interaction was characterised by determining whether surface-bound β protein directly binds LILRB3-expressing immune cells. The adherence of FITC-labelled A909 strains to U937 cell lines was measured by flow cytometry (**Fig 3.1F**). The fluorescence of the LILRB3-expressing U937 cell line to the U937 cell line for each A909 strain was normalised, to account for variation in FITC-labelling between A909 strains. The relative bacterial adherence of *S. agalactiae* strains to the LILRB3-expressing U937 cell line was then measured (**Fig 3.1F**). The data showed that β -expressing strains, A909 and $\Delta bac + pLZ.bac$, adhered significantly more to the LILRB3-expressing U937 cells than the Δbac strain (**Fig 3.1F**). Therefore, surface expressed β protein enhances cellular adhesion of *S. agalactiae* via LILRB3. Thus, the data suggest that β protein expressed on the *S. agalactiae* cell surfaces enables binding to LILRB3 expressed on immune cell surfaces.

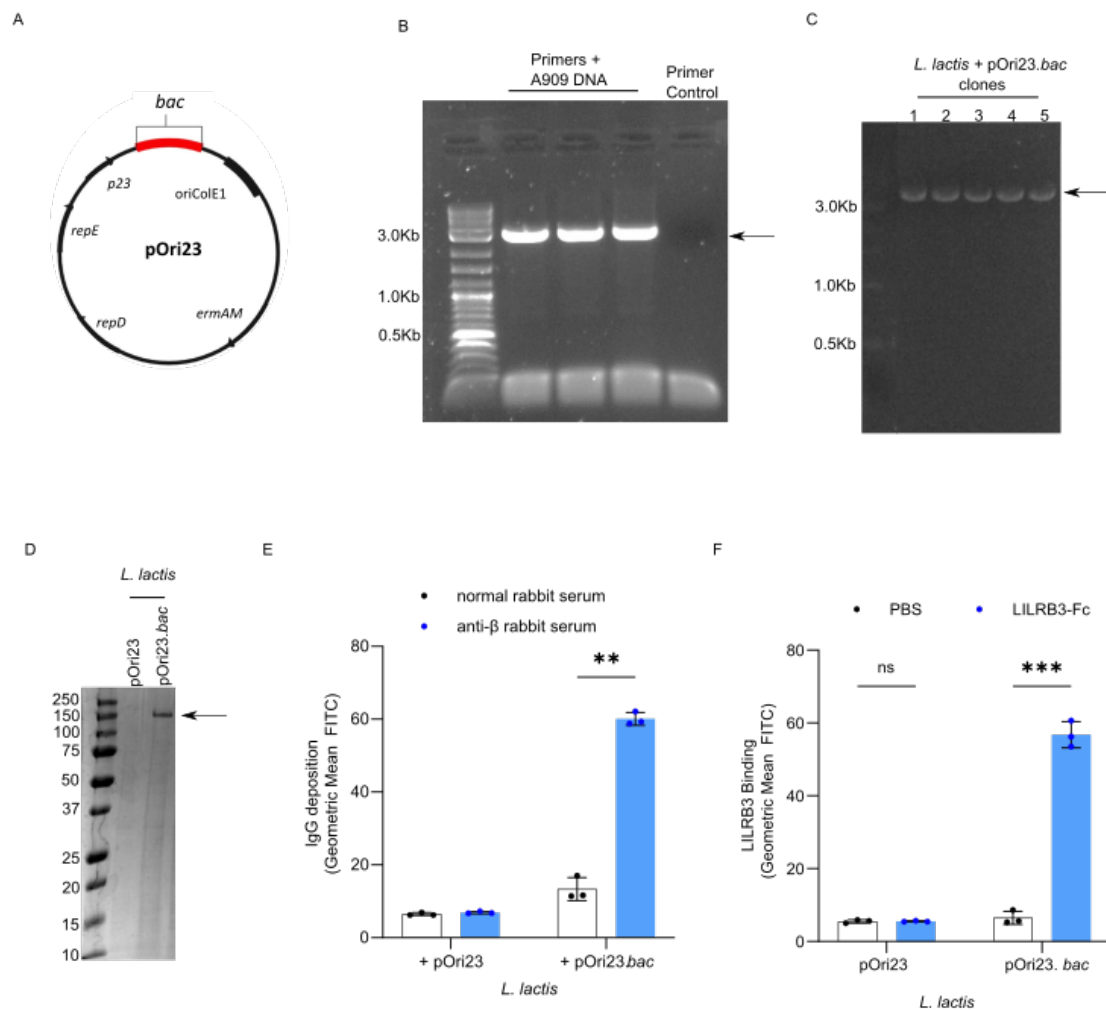


Figure 3.2. Surface β protein expression is sufficient for LILRB3 binding to bacterial surfaces. (A) Schematic showing β -expressing vector (pOri23.bac) used to transform *L. lactis*. **(B)** PCR amplification of *bac* gene from A909 DNA assessed by agarose gel. Arrow indicates band at predicted size for *bac* gene (3.4 kb) **(C)** Colony PCR was used to assess pOri23.bac vectors carried by *L. lactis* clones for presence of *bac* gene, measured by agarose gel. *L. lactis* samples were boiled at 95°C, and colony PCR using primers designed to amplify insert sequence (red band A). 5 clones are shown, with an arrow indicating *bac* insert. **(D)** Expression of β protein by *L. lactis* strains measured by SDS-PAGE analysis. Lysates of stationary phase isogenic *L. lactis* carrying pOri23 and pOri23.bac vectors, with an arrow indicating β protein band. **(E)** Surface expression of β protein of isogenic *L. lactis* strains, detected by incubation in 0.1% (v/v) anti- β or non-immune rabbit serum and flow cytometry analysis. **(F)** Binding of rLILRB3-Fc to *L. lactis* strains measured by flow cytometry. Stationary phase isogenic *L. lactis* strains were incubated with rLILRB3-Fc (10 μ g/mL) or PBS for 30mins, washed in PBS, and stained with goat anti-human-Fc-FITC mAb (1:1,000) for 30 mins. Samples were then washed in PBS, and fixed in 1% PFA All experiments were performed in at least three independent experiments with biological replicates shown for each data point, with the mean and SD shown. Two-way ANOVA was performed to assess levels of statistical significance (** $p < 0.01$, *** $p < 0.005$).

To assess whether β protein was sufficient to confer the LILRB3-binding of *S. agalactiae*, a *L. lactis* strain expressing surface β protein was constructed. *L. lactis* is a useful model to study streptococcal membrane

proteins because *L. lactis* is a Gram-positive bacterium for which there is a large variety of genetic tools and methods available (Frelet-Barrand 2022). An *E. coli*-*L. lactis* shuttle vector pOri23 containing a *bac* gene insert (pOri23.*bac*, **Fig 3.2A**) was constructed. The *bac* gene was amplified from A909 DNA (**Fig 3.2B**) and ligated into pOri23 previously digested with *Bam*HI and *Sall*. The transformants carrying the pOri23.*bac* vector were tested for the presence of the *bac* gene by colony PCR and by sequencing. The successfully assembled vector was subsequently transformed into *L. lactis*. The agarose gel showed that 5 transformed *L. lactis* colonies (*L. lactis* + pOri23.*bac*) carried pOri23 vectors with inserts at the expected size of the *bac* gene ~3.5kb (**Fig 3.2C**).

β protein expression of *L. lactis* strains was assessed by SDS-PAGE and anti- β serum deposition. SDS-PAGE analysis of *L. lactis* lysates showed that *L. lactis* carrying pOri23.*bac* highly expressed a protein at ~130 kDa, the predicted weight of β protein (**Fig 3.2D**). In contrast, *L. lactis* carrying pOri23 showed no expression, as expected. Furthermore, *L. lactis* carrying pOri23.*bac* expressed β protein on the cell surface, as determined by deposition of anti- β IgG onto the bacterial surface following incubation in anti- β sera (**Fig 3.2E**). Thus, a *L. lactis* strain that expresses β protein on its surface (pOri23.*bac*) was successfully constructed. To assess whether β protein was sufficient to confer a LILRB3-binding phenotype to *L. lactis*, I measured rLILRB3-Fc binding to *L. lactis* strains by flow cytometry (**Fig 3.2F**). rLILRB3-Fc bound to the β -expressing *L. lactis* strain, but not *L. lactis* carrying the empty vector. Therefore, surface β protein expression is both necessary and sufficient for LILRB3 binding to bacterial surfaces.

3.3.2 Engagement of LILRB3 by β protein causes LILRB3 cross-linking

Next, the consequences of β engagement of LILRB3 for immune cell signalling was investigated. As clustering of LILRB3 due to cross-linking is critical to propagate inhibitory immune signalling and regulating receptor-mediated effector functions in immune cells (Zhao et al. 2020), the hypothesis proposed was that β protein causes LILRB3 cross-linking, triggering intracellular signalling. To test this hypothesis, a 2B4T reporter cell line, previously constructed by the McCarthy lab (Zhao et al. 2020), that stably expresses LILRB3 receptors with CD3 ζ intracellular domains was utilised (**Fig 3.3A**). Crosslinking of CD3 ζ in 2B4T reporter cell lines causes dephosphorylation of NFAT, inducing translocation of NFAT to the nucleus and inducing gene expression of a green fluorescence protein (GFP). Thus, cross-linking of CD3 ζ promotes fluorescence. Wildtype 2B4T reporter cells and 2B4T LILRB3CD3 ζ reporter cell lines were incubated with A909, A909 Δ *bac* or PBS for 24 hours. The fluorescence of the reporter cell lines was subsequently measured by flow cytometric analysis (**Fig 3.3B**). The A909 strain induced significantly higher levels of GFP expression in the LILRB3CD3 ζ reporter cells compared with the control cell line. Further, A909 induced more GFP expression in the LILRB3CD3 ζ cell line compared to the Δ *bac* strain or PBS, suggesting that β protein expressed by A909 induces LILRB3 crosslinking (**Fig 3.3B**). Thus, these data suggest a novel mechanism that *S. agalactiae* utilises to induce inhibitory signalling in immune cells, namely via β -mediated LILRB3 intracellular signalling.

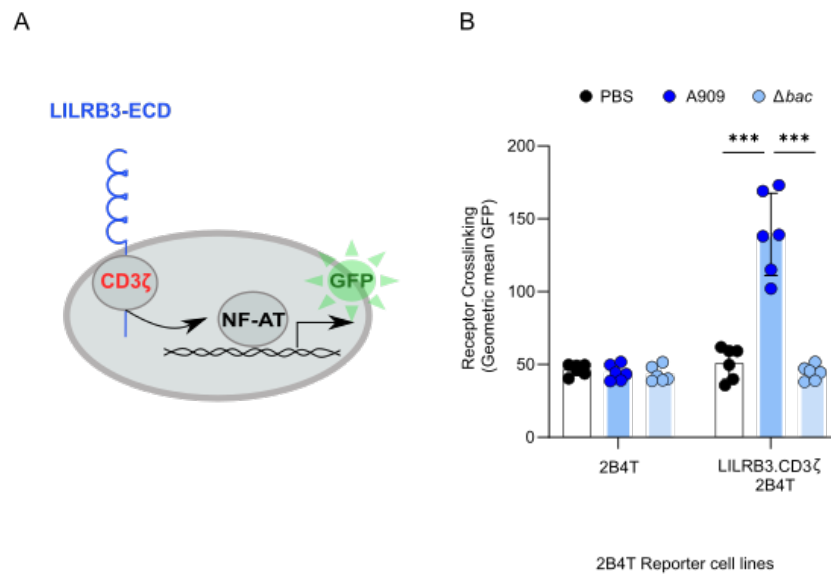


Figure 3.3. β protein induces cross-linking of LILRB3. (A) Schematic showing the 2B4T LILRB3CD3 ζ reporter cell line. The mechanism of fluorescence is highlighted, with the LILRB3 extracellular domain (LILRB3-ECD) in blue, CD3 ζ in red, NF-AT in black, and GFP in green. **(B)** A909 triggers crosslinking of LILRB3 via the β protein. Reporter cell lines wildtype or expressing LILRB3CD3 ζ were incubated with A909, Δbac A909, or PBS for 30mins, washed, then fluorescence was measured by flow cytometry. The assay was performed in three independent experiments, with repeats, mean and SD plotted. Statistical significance was assessed by 2-way ANOVA *** p < 0.005.

3.3.3 LILRB3 binds B6C and B75KN-CTD domains within the β protein

To understand the importance of β protein engagement of LILRB3 by *S. agalactiae* for immune evasion, it was first necessary to define the LILRB3 binding interface on the β protein. This is important because the β protein contains several well-defined domains that independently bind distinct immune-related host factors that may contribute to immune evasion as shown by the protein map (**Fig 3.4A**). B6N binds inhibitory immune receptors Siglec-5, Siglec-7 and Siglec-14 (Carlin et al. 2009; Ali et al. 2014; Fong et al. 2018, 7). IgI3 binds inhibitory receptors CEACAM1 and CEACAM5 (van Sorge et al. 2021). IgA domain nonopsonically binds IgA antibodies (Jerlström et al. 1996). Finally, B75KN binds to complement regulatory protein factor H (Areschoug et al. 2002). The multifunctionality of the β protein for binding immune-related factors prevents use of whole gene/protein knockout studies for elucidating the role of individual immune evasion mechanisms. Therefore, identifying the

LILRB3 binding domain was essential for developing the necessary tools to investigate the functional consequences of LILRB3- β protein interaction for immune evasion.

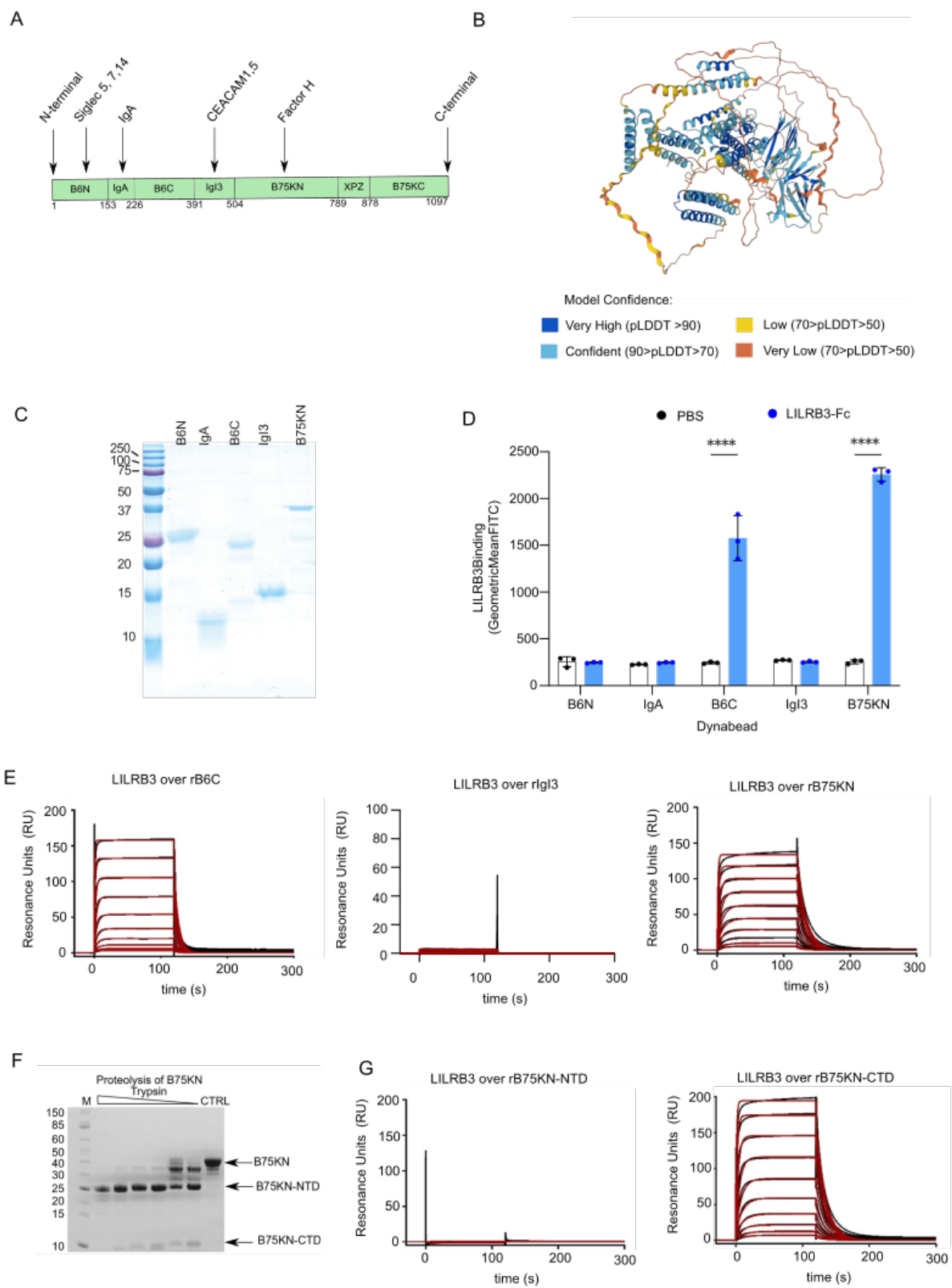


Figure 3.4. LILRB3 binds two independent domains in the β protein. (A) A schematic representation of the β protein with the size and location of domains drawn to scale. Host ligands known to interact with each domain are shown with arrows. (B) AlphaFold predicted structure of β protein (Accession: AF-P27951-F1). Prediction is coloured by model of confidence bands. (C) SDS-PAGE to assess the purity of His-tagged β protein domain proteins - B6N, IgABR, B6C, IgI3, and B75KN. (D) LILRB3 interaction with β protein domains was assessed by flow cytometry. β protein domain proteins were bound to HisTrap Dynabeads, then incubated with rLILRB3-Fc or PBS, washed, and stained for rLILRB3-Fc binding using anti-human-Fc-FITC mAb (1:1,000). Dynabead fluorescence was measured by flow cytometry. The assay was performed in three independent experiments, with the mean and SD used to determine the statistical significance by two-way ANOVA **** $p < 0.001$. (E,G) Reference-corrected SPR series for binding of LILRB3 to immobilized rB6C, IgI3, and B75KN (E) and B75KN-NTD and B75KN-CTD (G) showing both the experimental data (black traces) and the fit to a ligand heterogeneity model (red traces). (F) Limiting proteolysis of rB75KN by trypsin. SDS-PAGE analysis showing B75KN fragment (CTRL) after incubation with increasing concentrations of trypsin for 30 min. B75KN-NTD and B75KN-CTD fragments are highlighted by arrows. Molecular size standards (M) are shown on the left of the gel image. Polypeptide species at ~ 25 kDa, indicated by arrow, were sent for tryptic-peptide mass fingerprinting.

Defining the LILRB3 binding interface to a high resolution of within a few residues is difficult due to the large size of the β protein with it being composed of ~ 1000 residues. Therefore, initially LILRB3 binding to domains within the β protein was investigated. As there is currently no crystal structure for the β protein, a predicted model available on AlphaFold was used (Fig 3.4B). The AlphaFold model confidence is high for the β protein, with regions of high confidence (dark and light blue) generally being α helical structures localised nearer the centre of protein, whilst the regions of low predictive confidence (yellow and orange) are generally the loops located closer to the periphery of the protein (Fig 3.4B). Although, the AlphaFold model generated is useful for informing predictions of LILRB3 binding domains, especially due to the high confidence of the model, the size of the β protein and the high number of distinct structures still leaves many possible LILRB3 binding domains.

To identify the LILRB3 binding domains of β protein, a panel of β protein domains containing a His-tag (rB6N, rIgABR, rB6C, rIgI3, and rB75KN) that were previously expressed in *E. coli* (van Sorge et al. 2021) was tested. Purity of recombinant β protein domain proteins was assessed by SDS-PAGE (Fig 3.4C). Having established the high purity of the recombinant proteins, the binding of rLILRB3-Fc to Dynabeads coated in the recombinant β proteins was tested by flow cytometry (Fig 3.4D). rLILRB3-Fc showed statistically significant binding to rB6C- and rB75KN-coated Dynabeads, but not to rB6N-, rIgABR-, and rIgI3-coated Dynabeads. These data suggested that LILRB3 binds two independent domains within the β protein: B6C and B75KN. To confirm and explore these findings further, a distinct methodological approach of surface plasmon resonance imaging (SPR) was used. rLILRB3 was injected over immobilised rB6C, rIgI3, and rB75KN proteins, and the binding affinity measured (Fig 3.4E). rLILRB3 bound rB6C with a K_d of $400 \text{ nM} \pm 1.55$ and rB75KN with $KD \text{ (nM)} = 131 \text{ nM} \pm 0.77$. In agreement with previous findings, rLILRB3 showed no binding to rIgI3. These SPR data confirmed that B6C and B75KN are LILRB3 binding domains. They further revealed that LILRB3 has a 3 times stronger binding affinity for B75KN compared with B6C.

B75KN is a large domain formed of ~250 residues. Therefore, the LILRB3 binding was further localised within the B75KN domain. To assess whether B75KN could be separated into smaller stable fragments, limiting proteolytically cleavage of rB75KN domain using trypsin or subtilisin was performed that resulted into two stable domains: N-terminal domain (residues 504-687, B75KN-NTD) and C-terminal domain (residues 688-789 B75KN-CTD) (**Fig 3.4F**, repeated in Chapter 4). Utilising these co-ordinates, rB75KN-CTD and rB75KN-NTD were generated, and rLILRB3 was injected over immobilised rB75KN-CTD and rB75KN-NTD (**Fig 3.4G**). It was observed that rLILRB3 binds solely to the rB75KN-CTD domain.

In conclusion, two LILRB3 binding domains in the β protein were identified: B6C and B75KN. Within B75KN, the LILRB3 binding region localises to B75KN-CTD. Of note, B6C and B75KN-CTD bind LILRB3 independently of each other, with B75KN binding LILRB3 with ~3 times higher binding affinity. Having established two LILRB3 binding domains composed of ~100 residues, the AlphaFold predictive model of β protein (**Fig 3.4B**) could be used to identify candidate residues involved in LILRB3 binding and to define the LILRB3 binding interface.

3.3.4 Defining the LILRB3 binding interface on the β protein.

Having localised LILRB3 binding to B6C (magenta) and B75KN-CTD (yellow) domains (**Fig 3.5A-B**), next the residues that may be involved in LILRB3 binding were assessed. The hypothesis proposed was that key residues in the B6C and B75KN-CTD domains involved in LILRB3 binding would have side chains that form the surface of the molecule. To test this hypothesis, a series of rB6C and rB75KN proteins containing individual residues mutated were produced and then measured levels of LILRB3 binding compared with their corresponding wildtype. To increase the likelihood of selecting candidate residues involved in LILRB3 binding, residues with external facing side chains, based on the AlphaFold predicted structure (**Fig 3.5B**), across the entire B6C or B75KN-CTD domains were selected, with the aim of selecting at least one candidate residue for each loop and α helical structure. To produce a series of rB6C and rB75KN His tagged mutant proteins, site-directed mutagenesis was performed on vectors expressing wildtype rB6C or rB75KN His-tagged proteins to substitute each residue for an alanine residue at each selected candidate residue.

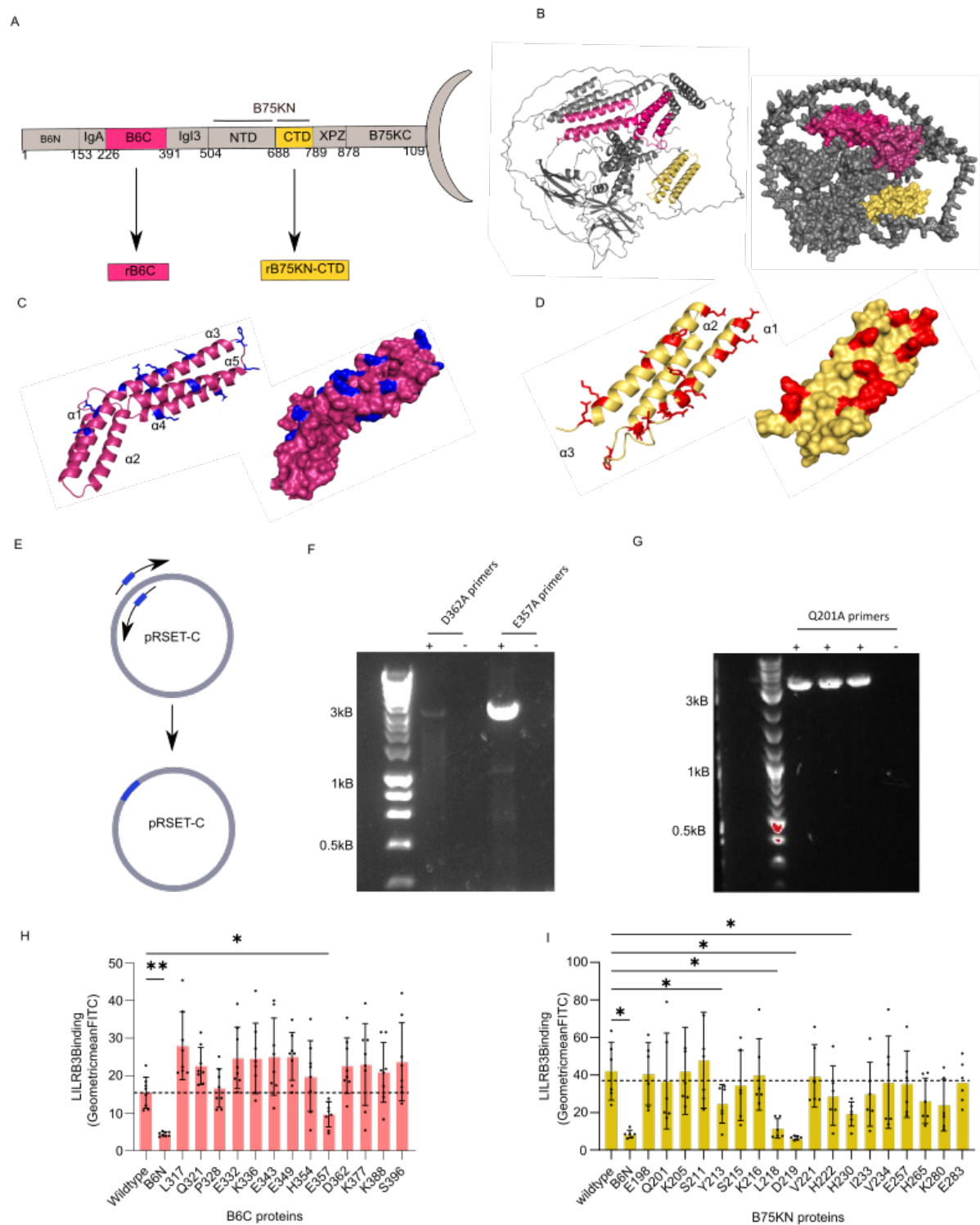


Figure 3.5. Defining the LILRB3 binding interface on the β protein. (A) A schematic representation of the β protein with two domains (B6C, and B75KN-CTD) highlighted. (B) AlphaFold predicted structure of β protein (Accession: AF-P27951-F1), shown in both cartoon representation (left panel) and surface representation (right panel). The B6C and B75KN-CTD regions are highlighted in magenta and yellow, respectively. (C-D) Cartoon and surface representations of B6C (C) and B75KN-CTD (D) with residues that were selected for site-directed mutagenesis in blue. (E) Schematic showing site directed mutagenesis by inverse PCR. (F-G) Agarose gel showing inverse PCR amplification of pRSET-C.B6C (F) and pRSET-C.B75KN (G) using primers designed to introduce alanine substitutions at specific residues. (+) denotes addition of pRSET-C vector and (-) buffer control. (H-I) rLILRB3-Fc binding to rB6C (H), and rB75KN (I) mutant proteins, as assessed by flow cytometry. A series of proteins were expressed that contained single mutations of residues to alanine. Each column is labelled by the number and type of amino acid residue mutated. Mutant proteins were bound to HisTrap Dynabeads (30 μ g/mL), then incubated with rLILRB3-Fc (10 μ g/mL) or PBS, washed, and stained for rLILRB3-Fc binding using anti-human-Fc-FITC mAb (1:1,000). Dynabead fluorescence was measured by flow cytometry. The assay was performed in at least three independent experiments, with repeats, mean and SD plotted. Statistical significance was assessed by one-way ANOVA * $p < 0.05$, ** $p < 0.01$.

Illustrated are the cartoon and surface structures of B6C (Fig 3.5C) and B75KN-CTD (Fig 3.5D), with the candidate mutated highlighted in blue and sticks representing the side chains. B6C is composed of 5 α helices (α 1-5) connected by 4 loops, oriented with a large α 3 helices flanked by smaller pairs of α 1 and α 2, and α 4 and α 5 helices in antiparallel orientation (Fig 3.5C). Site directed mutagenesis (SDM) by inverse PCR (Fig 3.5E) was used to produce a series of pRSET-C.B6C and pRSET-C.B75KN vectors that contained single alanine substitutions at specific residues (Fig 3.5F-G). Vectors were transformed into an *E. coli* protein expression system (RG) and rB75KN-His and rB6C-His mutant proteins were purified using His-trap columns and the Akta purification system.

13 B6C mutant proteins were produced with alanine substitutions at residues L317, Q321, P328, E332, K336, E343, E349, H354, E357, D362, K377, K388, or S396 (Fig 3.5C). This represented partial coverage of the B6C domain, with α 3 (L317, Q321, P328, E332, K336, E343, E349), loop 3 (H354, E357), α 4 (D362, K377), and α 5 (K388, S396) all being represented by at least two mutant proteins. However, we lacked coverage of residues at α 1 and α 2, and the 1st, 2nd, and 4th loops. To assess the involvement of individual residue side chains involved in LILRB3 binding to B6C, Dynabeads were coated with rB6C-His mutant proteins and rLILRB3-Fc binding by flow cytometry was measured (Fig 3.5H). rLILRB3-Fc binding to Dynabeads coated in wildtype rB6C (Fig 3.5H) acted as positive controls, and rB6N Dynabeads acted as a negative control. For all rB6C-His mutant proteins there was no statistically significant difference in LILRB3 binding compared to wildtype rB6C, except for the E357A mutation, which exhibited reduced LILRB3 binding (Fig 3.5H). Thus, the side chain of glutamic acid at residue 357 (E357) is likely important for LILRB3 binding to B6C.

B75KN-CTD is composed of 3 alpha helices (α 1-3) connected by 2 loops, with the helices in an antiparallel orientation (Fig 3.5D). 18 different B75KN mutant proteins were produced with alanine substitutions at residues E198, Q201, K205, S211, Y213, S215, K216, L218, D219, V221, H222, H230, I233, V234, E257, H265, K280, or E283 (Fig 3.5D). This represents full coverage of the B75KN-CTD domain, with proteins containing mutations in α 1 (E198, Q201, K205, S211, Y213, S215, K216, L218), 1st loop (D219, V221, H222, H230), α 2 (I233, V234), 2nd loop (E257), and α 3 (H265, K280, or E283). The same method for assessing LILRB3 interaction with B75KN proteins as for B6C proteins was utilised (Fig 3.5I). Wildtype B75KN acted as the positive control, and B6N as the

negative control. For B75KN, mutations at residues L218 or D219 abrogated LILRB3 binding, whilst mutations at Y213 and H230 reduced LILRB3 binding (**Fig 3.5I**). Thus, L218 and D219 are likely located at the LILRB3 binding interface, whilst Y213 and H230 residues may support the correct secondary structure folding required for LILRB3 binding (**Fig 3.5I**).

Interestingly, the putative LILRB3 binding interfaces that were identified for both B6C and B75KN-CTD are located on or near loops (**Fig 3.6A-B**). The B6C residue E357 is located on the 3rd loop that connects α 3 and α 4 (**Fig 3.6A**), whilst B75KN-CTD residues L218 and D219 are situated on the border of the 2nd loop connecting α 1 and α 2 helices, whilst Y213 and H230 are on nearby alpha helices (**Fig 3.6B**). Loops are highly flexible structures within proteins, that may allow for conformational changes required to bind LILRB3. The putative LILRB3 binding domains in B6C (**Fig 3.6C**, magenta) and B75KN-CTD (**Fig 3.6D**, yellow) are distally located to each other, suggesting that β protein may bind two LILRB3 receptors independently. Thus, the relatively large distance between LILRB3 binding interfaces on the β protein may ensure β protein binds two LILRB3 receptors, causing LILRB3 receptor clustering. This could be important because LILRB3 clustering on the surface of immune cells is key for triggering intracellular signalling and controlling immune cell functions (Zhou et al. 2023)

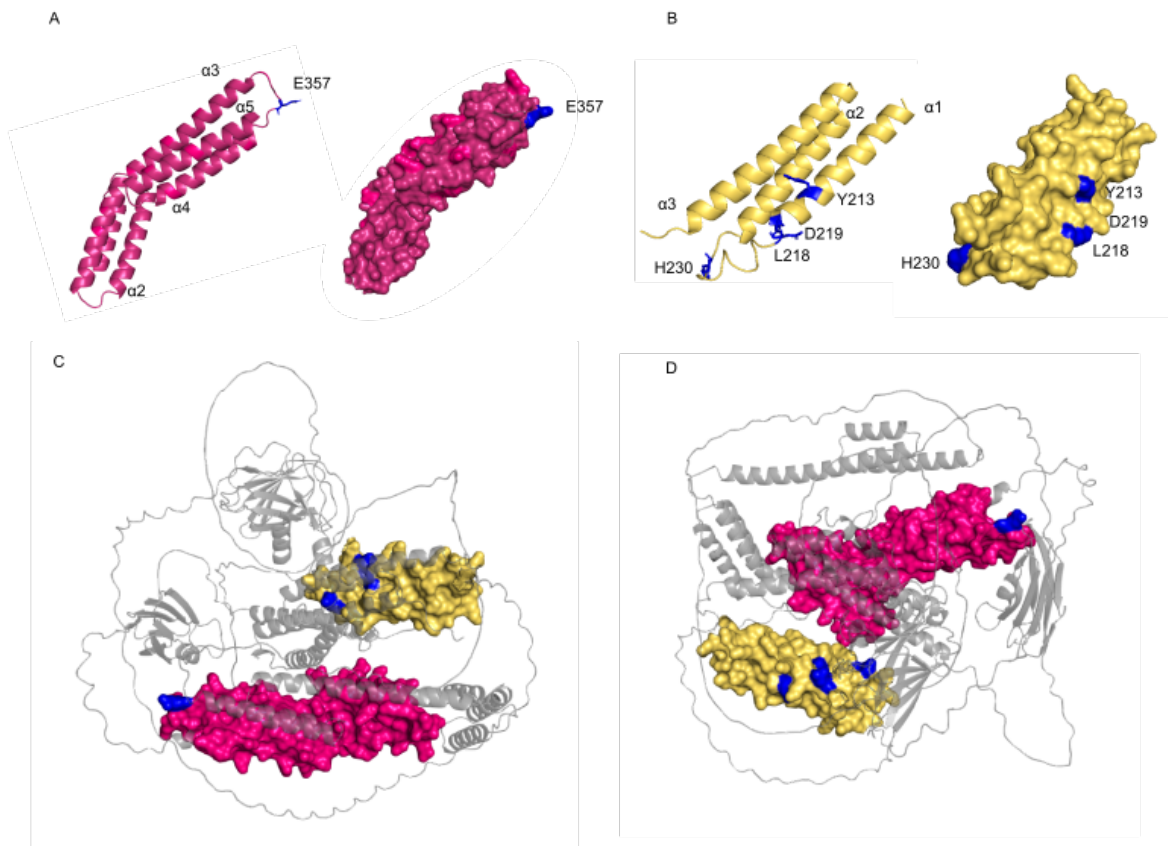


Figure 3.6. β protein residues involved in LILRB3 binding are distally located, suggesting a mechanism for LILRB3 clustering. (A-B) Cartoon and surface representations of B6C (A) and B75KN-CTD (B) with residues important for LILRB3 binding highlighted in blue. **(C-D)** Two orientations of AlphaFold predicted structures of β protein (Accession: AF-P27951-F1) showing surface representation of B6C and B75KN-CTD and cartoon for all other domains. The B6C (magenta), B75KN-CTD (yellow), and key LILRB3 binding residues (blue) are highlighted.

3.3.5 Constructing β -expressing *L. lactis* with E357 and L218 residues mutated

Having identified two residues – E357 and L218 – that are important for LILRB3 binding to B6C and B75KN-CTD domains, respectively, the next aim was to construct a *L. lactis* strain that expressed full length β protein with these residues mutated. β protein is multifunctional and interacts with several immune factors that may play a role in immune evasion. Therefore, it was necessary to construct *L. lactis* strains that expressed a β protein with E357 and L218 residues mutated to alanine ($\beta\Delta E357\Delta L218$) to specifically abrogate LILRB3 binding whilst retaining the capacity to interact with other immune factors. To this end, two consecutive SDM experiments were performed using pOri23.bac as the template vector to construct a pOri23.bac $\Delta E357\Delta L218$ vector (**Fig**

3.7A). Initially, SDM by inverse PCR was performed using pOri23.*bac* as the template vector and E357A primers to produce pOri23.*bac*ΔE357 with amplification assessed by agarose gel (**Fig 3.7B**). After confirmation of the mutation in the vector by sequencing, pOri23.*bac*ΔE357 was subsequently used as the template vector for SDM using L218 primers, to produce pOri23.*bac*ΔE357ΔL218 vector (**Fig 3.7C**). The final constructed vectors were sent for full plasmid sequencing to confirm correct mutations.

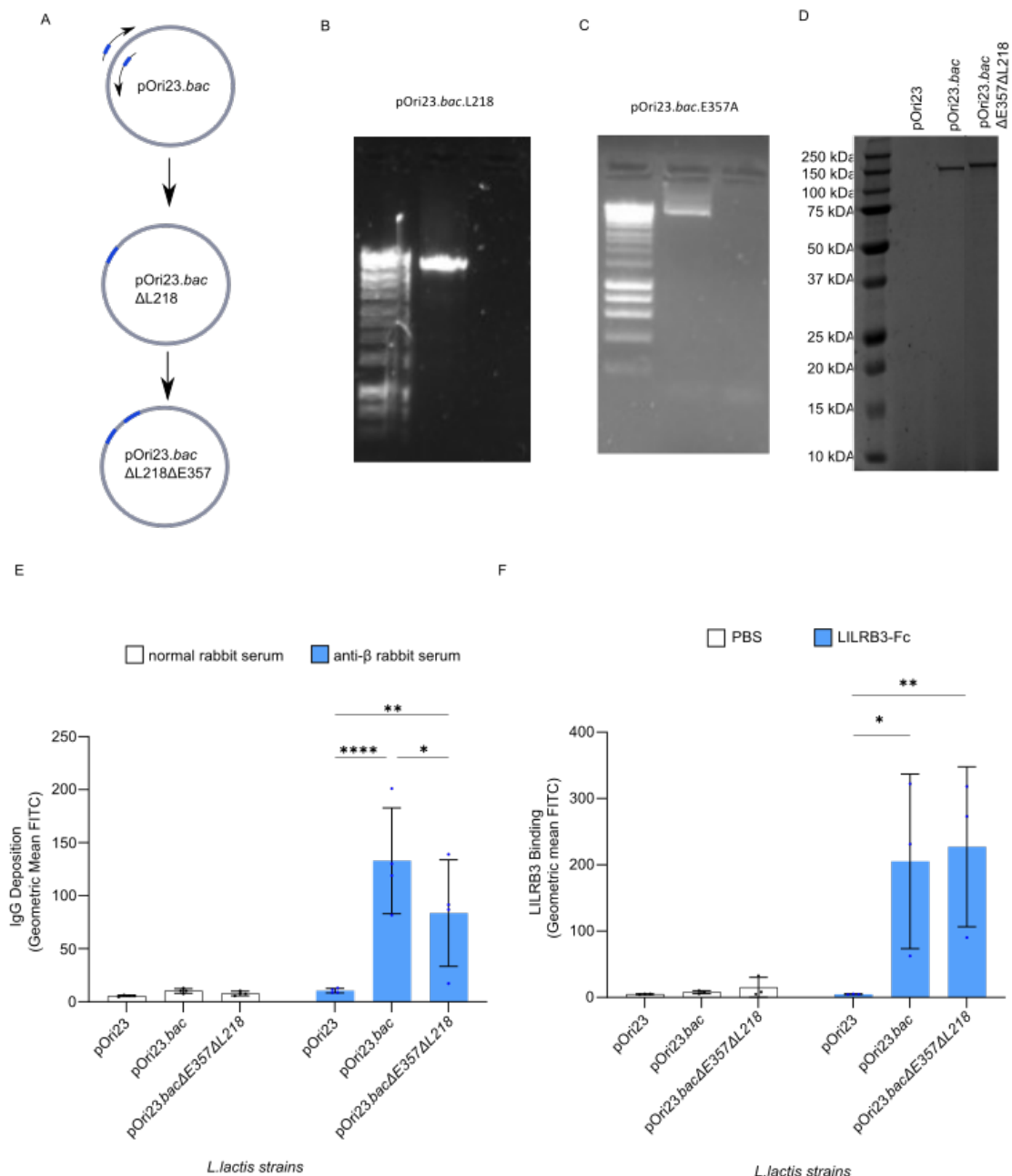


Figure 3.7. Mutation of E357 and L218 residues on β protein is not sufficient to abrogate LILRB3 binding. (A) Schematic of SDM inverse PCR used to produce pOri23.*bac* Δ E357 Δ L218 vector. (B-C) SDM of pOri23.*bac* (B) and pOri23.*bac* Δ E357 vectors using E357A and L218A primers, respectively. (C) Vectors assessed by agarose gel. (D) Expression of β protein by *L. lactis* carrying pOri23.*bac* Δ E357 Δ L218, as measured by SDS-PAGE analysis. Lysates of stationary phase isogenic *L. lactis* carrying pOri23, pOri23.*bac* or pOri23.*bac* Δ E357 Δ L218 vectors. (E) Surface expression of β protein by isogenic *L. lactis* strains, detected by incubation in 0.1% (v/v) anti- β or non-immune rabbit serum and flow cytometry analysis. (F) Binding of rLILRB3-Fc to *L. lactis* strains measured by flow cytometry. Stationary phase isogenic *L. lactis* strains were incubated with rLILRB3-Fc (10 μ g/mL) or PBS for 30mins, washed in PBS, and stained with goat anti-human-Fc-FITC mAb (1:1,000) for 30mins. Samples were then washed in PBS, and fixed in 1% PFA. All experiments were performed in at least three independent experiments with biological replicates shown for each data point, with the mean and SD shown. Two-way ANOVA was performed to assess levels of statistical significance (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.001$).

Having verified that the sequence of pOri23.*bac* Δ E357 Δ L218 vector was correct, the vector was transformed into *L. lactis* and total protein expression of β protein was measured by SDS-PAGE (Fig 3.7D). *L. lactis* carrying pOri23.*bac* Δ E357 Δ L218 produced a band at a similar size as pOri23.*bac* (130kDa), demonstrating that β protein is expressed (Fig 3.7D). Of note, there was a slight shift in β protein size between pOri23.*bac* and pOri23.*bac* Δ E357 Δ L218, which was surprising given that only two residues were mutated. The Δ E357 Δ L218 mutations introduced into the β protein may have increased dimersation of the mutant β protein, which may explain the apparent higher protein size of β Δ E357 Δ L218. To assess whether β Δ E357 Δ L218 was surface expressed by *L. lactis*, anti- β serum deposition was measured by flow cytometry (Fig 3.7E). *L. lactis* expressing β Δ E357 Δ L218 (pOri23.*bac* Δ E357 Δ L218) had significantly higher levels of anti- β IgG deposition than empty vector control (pOri23) demonstrating that β Δ E357 Δ L218 is expressed on the surface of *L. lactis* (Fig 3.7E). Interestingly, *L. lactis* carrying pOri23.*bac* had significantly more anti- β IgG deposition than the pOri23.*bac* Δ E357 Δ L218, suggesting *L. lactis* carrying pOri23.*bac* may express higher levels of surface β protein. Alternatively, the lower IgG deposition exhibited by pOri23.*bac* Δ E357 Δ L218 may be due to the Δ E357 Δ L218 mutations inducing conformational changes in the β protein, that result in reduced IgG deposition. Finally, rLILRB3-Fc binding to *L. lactis* expressing β Δ E357 Δ L218 was tested by flow cytometry (Fig 3.7F). Surprisingly, rLILRB3-Fc binding to *L. lactis* expressing β Δ E357 Δ L218 was not significantly different to wildtype β -expressing *L. lactis* (pOri23.*bac*), suggesting that mutation of E357 and L218 residues is not sufficient to abrogate LILRB3 binding to β protein (Fig 3.7F). Therefore, mutation of additional β protein residues is required to abrogate LILRB3 interaction. This could potentially involve residues such as D219, H230, and Y213 that were identified as forming the LILRB3 binding interface in the previous biochemical assays.

3.3.6 Blocking β protein binding to LILRB3 enhances neutrophil phagocytosis of *L. lactis*

As the *L. lactis* strain expressing $\beta\Delta E357\Delta L218$ maintained LILRB3 interaction, this hampered investigations into the functional consequences of the LILRB3- β protein interaction. Despite this, a preliminary neutrophil phagocytosis assay was performed to develop a protocol for the functional consequences of LILRB3- β interaction in immune evasion (**Fig 3.8**). As an alternative means, I competitively blocked LILRB3 engagement with wildtype β protein expressing *L. lactis* (pOri23.bac) by preincubation of the bacteria with rLILRB3-His prior to infection of the neutrophil culture. These data showed that preincubation of *L. lactis* expressing β protein with rLILRB3-His enhances neutrophil phagocytosis compared with PBS control (**Fig 3.8**). Unfortunately, insufficient time prevented additional performing additional repeats of this assay and further replicates are required to confirm this result. Despite this, these data potentially indicate that β protein engagement with LILRB3 expressed on the surface of neutrophils suppresses neutrophil phagocytosis.

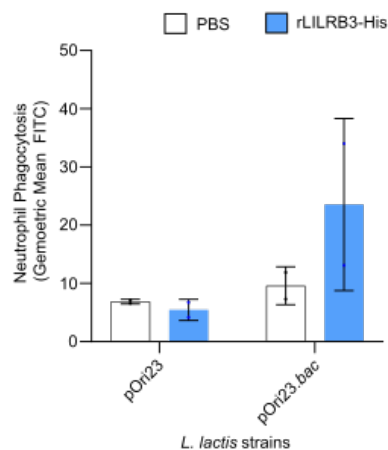


Figure 3.8. β protein interaction with LILRB3 suppresses neutrophil phagocytosis. Phagocytosis of FITC-labelled *L. lactis* strains by human neutrophils. FITC-labelled stationary phase *L. lactis* strains were preincubated with rLILRB3-His or PBS for 30 mins, washed, and then incubated with human neutrophils at a multiplicity of infection of 10 for 30 min at 37°C with shaking. Neutrophils were stained with trypan blue for 5 mins to quench extracellular bacteria, washed and fluorescence measured by flow cytometry. Mean and SD were plotted and two-way ANOVA was performed to assess statistical significance of two independent experiments.

3.4 Discussion

In this study, the *S. agalactiae* β protein was identified as a ligand for the inhibitory immune receptor LILRB3. Surface expression of β protein was shown to be both necessary and sufficient for *S. agalactiae* to bind to LILRB3. β protein is a large 130kDa cell-wall anchored protein encoded by the *bac* gene that is carried predominantly

by virulent *S. agalactiae* strains Ia, Ib, and II serotypes, and less frequently by III and V serotypes (Dore et al. 2003; Manning et al. 2006; Carlin, Chang, et al. 2009). As β protein expression is associated with invasive *S. agalactiae* disease, β protein clearly has a role in virulence. However, the mechanism through which β protein confers its virulence phenotype to *S. agalactiae* is not clear.

β protein possesses a remarkable ability to interact with multiple components of the immune system (**Fig 3.5A**), to subvert immune responses, and to promote bacterial survival in mammalian hosts. Indeed, surface-expression of β protein enables *S. agalactiae* to bind key components of the immune system, including IgA, FH, Siglec receptors and CEACAM receptors [Areschoug et al. 2002; Pleass et al. 2001; Fong et al. 2018; van Sorge et al. 2021]. For example, β protein has been shown to directly bind to IgA, blocking IgA interaction with CD89 receptors expressed on neutrophils, thus inhibiting IgA-mediated effector functions (Pleass et al. 2001). It has also emerged that β protein directly engages surface-expressed immune receptors: CEACAM1, CEACAM5 (van Sorge et al. 2021), Siglec-5, Siglec-14 (Ali et al. 2014; Carlin et al. 2009) and Siglec-7 (Fong et al. 2018). Whilst all of these receptors are known to modulate immune cell effector functions (Favier 2016), determining the exact role for immune evasion of β protein engagement with each of these receptors remains unclear due to the number of interactions formed. Previous studies showed that isogenic β protein knockout *S. agalactiae* strains display increased susceptibility to NK cell (Fong et al. 2018) and neutrophil killing (Carlin et al. 2009). The enhanced survival of β expressing *S. agalactiae* was attributed to the capacity of β protein to interact with inhibitory Siglec receptors. However, this must now be reconsidered as β protein is also known to interact with CEACAM1 and LILRB3. Thus, the specific receptors or factors that are responsible for enhancing immune evasion of *S. agalactiae* remains unclear, as the previous studies are confounded by the multifunctionality of the β protein and its ability to engage multiple factors involved in the immune response.

To specifically interrogate the importance of β protein engagement with LILRB3 for *S. agalactiae* immune evasion first requires precise mutagenesis of LILRB3-binding domains in β protein and a comparison of its properties with wildtype β protein. Here, both β protein domains B6C and B75KN were shown to have the capacity to bind LILRB3. This raises the question, why does β protein contain two LILRB3-binding domains that are separated by ~ 400 residues? This could be explained by the fact that two binding domains would enable cross-linking of LILRB3 which is required to induce a strong inhibitory signal in immune cells. Indeed, our data show that surface expressed β protein induces LILRB3 cross-linking using an 2B4T reporter cell line. Another reason for the multiple LILRB3-binding domains of β protein could be because LILRB3 binding is critical to maintain function. Alternatively, the presence of two structurally different LILRB3 binding domains may increase the number of LILRB3 variants that β protein can bind. LILRB3 is a highly polymorphic gene with a recent study detecting 32 nonsynonymous SNPs in LILRB3 genes from 91 individuals of European descent that express 1 or more LILRA6 genes (Bashirova et al. 2022). Therefore, one LILRB3 domain may not bind all LILRB3 variants, whilst expression of two structurally different LILRB3 binding domains increases the diversity of LILRB3 variants that β protein can bind. Expression of two LILRB3 binding domains by β protein supports the view of the importance of LILRB3 interaction, whether it be to maintain LILRB3 cross-linking or to increase the possible number of LILRB3

variant interactions. To understand the importance of LILRB3 interaction with β protein for bacterial immune evasion, knocking out or precise mutagenesis of the LILRB3 binding domains B6C and B75KN-CTD is required.

The B6C and B75KN-CTD LILRB3 binding interfaces were characterised to better understand the mechanism of LILRB3 interaction and enable development of tools to specifically interrogate LILRB3-mediated immune evasion. By screening a panel of B6C and B75KN-CTD mutant proteins for LILRB3 binding, it was demonstrated that residues E357 of B6C and residues L218, D219, H230 and Y213 of B75KN-CTD are important for LILRB3 binding. Interestingly, these residues are clustered around protein loops: the 3rd loop of B6C connecting α 3 and α 4, and the 1st loop of B75KN-CTD connecting α 1 and α 2. Protein loops are highly versatile structures that are essential for many ligand-protein interactions. Whilst mutation of individual residues E357 and L218 abrogated binding of LILRB3 to recombinant B6C and B75KN-CTD, respectively, mutations of E357 and L218 did not abrogate binding of LILRB3 to surface-expressed β protein. This was unexpected and it suggests that additional residues must be mutated to abrogate the interaction. To test this in future work, additional mutations should be introduced in the residues neighbouring E357 and L218 thereby attempting to disrupt the protein loops sufficiently to abrogate LILRB3 binding. Importantly, additional assays are required to rule out the possibility that alanine substitutions introduced at E357 of B6C and L218, D219, H230 and Y213 of B75KN-CTD have not disrupted the overall native conformation, thereby abrogating LILRB3 binding. To ensure that loss of LILRB3 binding in these mutant proteins is due to disruption of a LILRB3 binding domain and not loss of overall protein structure, additional assays are required using techniques such as circular dichroism to compare the structure of the B6C and B75KN-CTD mutant proteins with their respective wildtype proteins. Determining the LILRB3 binding interface on β protein will also be important for identification of the β binding domain on LILRB3.

Preliminary functional assays suggest that β protein engagement with LILRB3 expressed on neutrophils suppresses neutrophil phagocytosis. β protein expressing *L. lactis* that was blocked for LILRB3 interaction showed increased levels of neutrophil phagocytosis compared with PBS control. Whilst further repeats are required to determine whether differences observed are statistically significant, these data suggest that β protein expressed by *S. agalactiae* targets LILRB3 to suppress neutrophil phagocytosis. In support of this, β protein induces cross-linking of LILRB3. Cross-linking of LILRB3 has been shown to suppress ROS production and phagocytosis in neutrophils and neutrophil-like cell lines (Zhao et al. 2020). As β protein also engages inhibitory immune receptors CEACAM1 and Siglec-5 on neutrophils (Carlin et al. 2009; Fong et al. 2018; van Sorge et al. 2021), the engagement of β protein with LILRB3 may act synergistically to drive inhibitory signalling and promote *S. agalactiae* survival from neutrophil attack.

In conclusion, this work has identified that β protein from *S. agalactiae* is a ligand for LILRB3 and it has determined the LILRB3 binding interface on β protein. Furthermore, the data demonstrate that β protein can induce inhibitory signalling in immune cells and may suppress neutrophil phagocytosis. In future work, the construction of *L. lactis* β protein expressing strains with the LILRB3 binding interface disrupted and their implementation in functional assays including neutrophil killing, whole blood killing and monocyte/macrophage phagocytosis assays could provide opportunities for elucidating the biological properties of the β -LILRB3 interaction in *S. agalactiae* infection biology.

Chapter 4. Characterising *S. agalactiae* β protein binding to Factor H and its contribution to immune evasion

Chapter 4 is based on a paper published in the Journal of Immunology with collaborators at Kansas State University entitled “Group B *Streptococcus* Surface Protein β : Structural Characterization of a Complement Factor H: Binding Motif and Its Contribution to Immune Evasion”. The structural work was performed by Xin Xu and supervised by Brian Geisbrecht, whilst my research focused on the functional work.

4.1 Introduction

A key component of the innate immune response against bacteria is the complement system. Complement is crucial for recognising foreign pathogens and mounting an effective immune response through opsonisation of pathogens, triggering immune cell activation, and direct cytolysis. The importance of complement for bacterial clearance during infections is demonstrated by patients with mutations in complement genes such as C3 being more susceptible to severe and recurrent bacterial infections (Ram, Lewis, and Rice 2010). The complement system is composed of 3 arms: the classical, alternative and lectin pathways. Each pathway is activated by different stimuli which trigger proteolytic cascades that result in clearance of pathogenic microbes (Mogensen 2009). The C1q molecule of the classical pathway recognises immune-complexes and pathogen-associated molecular patterns (PAMPs) such as LPS, whilst Mannose-Binding Lectin (MBL) and ficolins that characterise the lectin pathway recognise carbohydrates (Merle et al. 2015). In contrast, the alternative pathway is spontaneously activated by hydrolysis of a thioester bond in C3 and is constitutively active at low levels in healthy individuals in a process known as tick-over (Merle et al. 2015). All pathways eventually funnel into the alternative pathway.

Activation of the alternative pathway is crucial for clearance of pathogenic microbes. C3b deposition on microbial surfaces is required for (1) opsonisation of microbes for clearance by circulating phagocytic immune cells such as neutrophils, and (2) formation of the membrane attack complex (MAC) that produces cytotoxic pores on microbial surfaces (Noris and Remuzzi 2013; Merle et al. 2015). The alternative pathway is initiated by hydrolysis of C3 to produce C3b and C3a (**Fig 4.1B**). C3b binds to microbial surfaces, whilst C3a is a potent anaphylatoxin that recruits immune cells to sites of infection and promotes inflammation (Gao, Cui, and Zhao 2020). Crucially, the alternative pathway contains an amplification loop that is essential for a robust

inflammatory response by triggering a complement cascade (**Fig 4.1B**) (Mogensen, 2009). The amplification loop is caused by the formation of the alternative pathway C3 convertase (C3bBb), in which surface-bound C3b forms a complex with factor B (FB), that allows for cleavage of FB by factor D (FD) into Bb, thus forming C3bBb. Once C3bBb is formed, all nearby C3 is hydrolysed to C3b and C3a, in turn producing further C3bBb, C3b, and C3a. This positive feedback loop enhances the complement cascade, C3b deposition on microbes, immune cell recruitment, and inflammation (**Fig 4.1B**). Thus, the alternative pathway remains constantly active whilst C3b is present with the pathway only being halted by inactivation of C3b. C3b is inactivated by factor I that, together with its co-factor factor H (FH), proteolytically cleaves C3b into the inactivate form iC3b (**Fig 4.1B**). Though C3b deposition is critical for targeting and directly lysing pathogenic microbes, C3b also binds host structures and therefore must be tightly regulated by complement regulatory factors to avoid damage to host tissues (Noris and Remuzzi 2013).

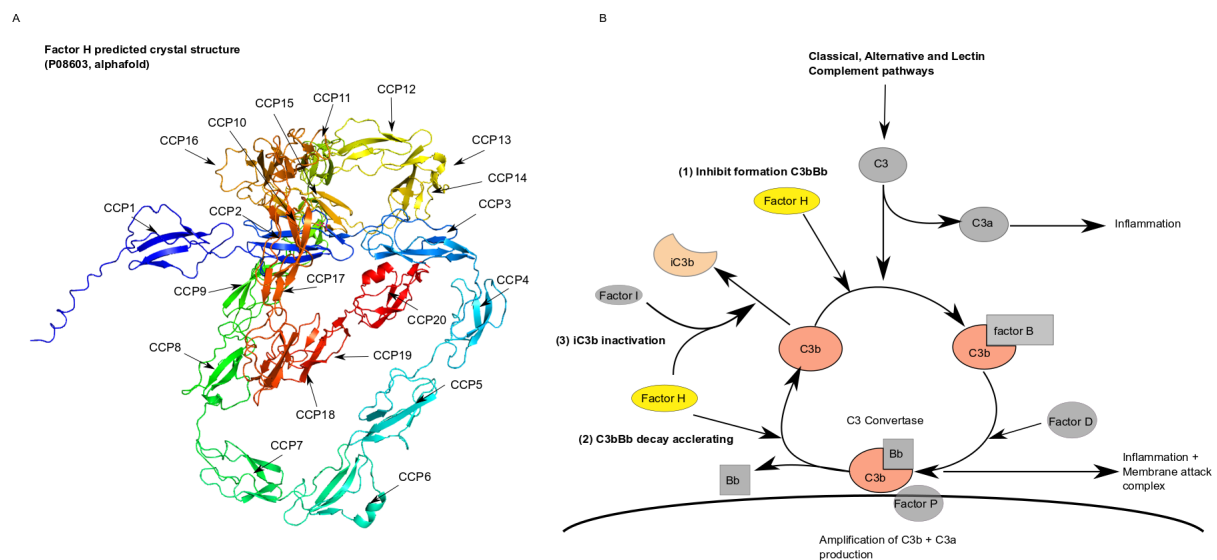


Figure 4.1. FH is a major complement regulator. (A) FH predicted crystal structure (AlphaFold; P08603) shown using a rainbow spectrum going from the C terminal in blue to the N-terminal in red. In addition, the 20 CCP domains are indicated by arrows. CCP domains are key for binding to host cell surfaces and key complement factors such as C3b. CCP1-4 are important for binding C3b and C3bBb convertase, whilst CCP6-7, and CCP18-20 are important for binding glycosaminoglycans on host cells (B) Schematic representation showing alternative complement pathway and the multiple mechanisms FH employs to inhibit this process. The alternative pathway is initiated by hydrolysis of C3 into C3b and C3a. C3b binds factor B which allows for factor D to proteolytically cleave factor B into Bb, thus producing C3bBb convertase which is anchored to the cell surface by factor P. C3bBb convertase can then produce more C3b in a positive feedback loop. FH inhibits the alternative pathway by (1) inhibiting formation of C3bBb convertase, (2) accelerating the decay of C3bBb convertase, or (3) acting as co-factor for factor I to proteolytically cleave C3b into the inactivate form iC3b.

The human immune system contains several negative regulators of complement that tightly control activation of the alternative pathway to prevent inappropriate inflammation and damage to host cells and tissues. FH is

the major fluid-phase complement regulator in blood due to its solubility, potency, and abundance (Pouw et al. 2016). Whilst FH naturally acts as a fluid-phase complement inhibitor, crucially FH binds host cell surfaces and extracellular structures to prevent inappropriate complement activation.

FH is composed of 20 complement control protein (CCP) domains, each containing ~60 amino acids, with the overall structure resembling a string of pearls (**Fig 4.1A**). Whilst these CCP domains are homologous in structure, specific CCPs perform distinct functions: CCP1-4 are important for binding C3b and C3bBb convertase, whilst CCP6-7 and CCP18-20 are important for binding glycosaminoglycans on host cells (**Fig 4.1A**). FH functions by reducing C3b deposition through (1) inhibiting formation of C3bBb convertase, (2) accelerating decay of C3bBb convertase, (3) acting as a co-factor for factor I (FI)-mediated proteolytic inactivation of C3b (**Fig 4.1B**). Through these three distinct mechanisms FH drastically reduces C3b deposition and C3bBb activity on host cell surfaces, thereby, inhibiting complement and reducing inflammation.

Several human bacterial pathogens sequester FH via surface adhesins to inhibit the complement cascade (Lambris, Ricklin, and Geisbrecht 2008). These include: M protein, Fba, and Scl1 (*S. pyogenes*); ShtI, ShtII, and β protein (*S. agalactiae*); PspC, Tuf, and LytA (*S. pneumoniae*); FHbp, NspA, PorB2, and PorB3 (*N. meningitidis*); CspZ, CspA, ErpP, ErpC, ErpA, and ErpN (*B. burgdorferi*); Sbi and SdrE (*S. aureus*) (Moore et al. 2021). The importance for pathogenic bacteria to sequester FH and suppress complement is highlighted by the numerous FH binding proteins that each species expresses. Furthermore, these FH binding proteins are structurally diverse, suggesting convergent evolution as a result of strong selective pressure for FH binding (Józsi 2017). Indeed, sequestering FH may contribute to the specificity of certain human pathogens; for example, *S. pneumoniae*, whose natural reservoir is the human nasopharynx, expresses PspC that specifically binds human FH but not FH from other animal species (Lu et al. 2008).

Whilst there is convincing evidence that human pathogenic bacteria sequester FH to suppress complement activation, whether this is required for immune evasion remains largely unclear. A common approach to investigate the role of FH sequestration for immune evasion by bacteria has been to compare the immune response to isogenic FH-binding and -nonbinding bacterial strains, typically using entire gene knockout mutant strains. These studies have demonstrated the importance of FH binding proteins for bacteria to resist opsonophagocytosis and enhance their survival in whole blood (Jarva et al. 2002; Haupt et al. 2008; Pian et al. 2012; Sharp et al. 2012). However, these results are confounded by the fact that (1) FH binding proteins are multifunctional often having multiple immune-related functions (Grosskinsky et al. 2009; Lesiak-Markowicz et al. 2011), and (2) bacteria often express multiple FH binding proteins (Moulin et al. 2019; Xu et al. 2022). Indeed, FH binding proteins often contain multiple domains with distinct structures that are able to independently bind complement-related and/or immune-related factors, making deciphering the precise role of FH interaction for immune evasion difficult to discern (Jarva et al. 2002; Haupt et al. 2008; Pian et al. 2012; Sharp et al. 2012). Therefore, whilst knockout studies using isogenic mutant *S. aureus* showed that Sbi inhibits opsonophagocytosis and that it is important for immune evasion, the antibody-mediated or FH-mediated properties have not been independently assessed (Haupt et al. 2008). The second confounding factor is that many bacterial strains express multiple FH binding proteins, which means that the functional effects of FH recruitment by one protein may be

masked by expression of other FH binding ligands. For example, *S. aureus* not only uses Sbi to bind FH but can also express SdrE that also binds FH (Sharp et al. 2012). In summary, the specific role of FH sequestration for immune evasion in bacteria remains unclear because of the multifunctionality of FH binding proteins, and the multiple FH binding proteins expressed by the strains tested.

S. agalactiae is a master complement inhibitor that expresses ShtI, ShtII and β proteins that are capable of independently sequestering FH. Whilst ShtI and ShtII proteins are ubiquitously expressed in human *S. agalactiae* strains, with 96.8% carrying the *shtI* gene and 100% carrying the *shtII* gene, β protein is only expressed by a subset of virulent serotypes of *S. agalactiae*. Previous studies investigating β protein have identified several distinct domains that independently bind immune inhibitory receptors LILRB3 (as shown in Chapter 3), Siglec-5, Siglec-7, and CEACAM1, and immune-related factors such as IgA (Carlin et al., 2009; Fong et al., 2018; van Sorge et al., 2021). Furthermore, β protein has been shown to interact with FH; however, the molecular mechanism of complement suppression and how this relates to immune evasion are poorly understood (Areschoug et al. 2002; Jarva et al. 2004). Investigations into β -mediated FH sequestration for immune evasion are hampered by *S. agalactiae* expressing multiple FH binding proteins and the multifunctionality of β protein.

4.1.1 Hypothesis and aims

The hypothesis that β protein sequesters FH to inhibit complement activation and enhance bacterial immune evasion was tested. To ascertain the function of β -mediated sequestration of FH, the aims were to (1) characterise the binding interface of FH and β protein, utilising biochemical and structural approaches, (2) utilise this knowledge to produce an appropriate model for specifically interrogating β sequestration of FH, and (3) determine the functional consequences for FH sequestering on complement and immune evasion using cellular and biochemical assays. The Geisbrecht lab determined the β -FH binding interface, whilst the McCarthy lab focused on producing a cellular model to determine the functional consequences of β -FH interaction for complement inhibition and immune evasion.

4.2 Material and methods

4.2.1 Bacterial strains and growth conditions

S. agalactiae (wild-type, Δbac , and Δbac + pLZ.*bac*) and the parental *L. lactis* MG1363 strain (**Table 4.1**) have been previously described (Areschoug et al. 2002; Wegmann et al. 2007). *S. agalactiae* was cultured at 37°C in

Todd Hewitt broth (THY), and for growth of the A909 Δ *bac* + pLZ.*bac* strain, THY was supplemented with 70 μ g/ml spectinomycin. *L. lactis* was cultured at 30°C in M17 broth supplemented with 0.5% (w/v) glucose and 0.5% (w/v) lactose (GM17) and strains with pOri23 were cultured in GM17 supplemented with 5 μ g/ml erythromycin.

Strain	Vector	Notes	Reference
<i>Streptococcus agalactiae</i>			
A909		Serotype Ia; <i>bac</i> positive	(van Sorge et al. 2021)
A909 Δ <i>bac</i>		A909 with <i>bac</i> gene deleted	(Areschoug et al. 2002)
A909 Δ <i>bac</i> + pLZ. <i>bac</i>	pLZ. <i>bac</i>	A909 Δ <i>bac</i> expressing surface β protein	(Areschoug et al. 2002)
<i>Lactococcus lactis</i>			
H1186 + pOri23	pOri23	H1186 carrying pOri23	Striskandan
MG1363 + pOri23. <i>bac</i>	pOri23. <i>bac</i>	MG1363 expressing surface β protein	This study
MG1363 + pOri23. <i>bac</i> Δ B75KN	pOri23. <i>bac</i> Δ B75KN	MG1363 expressing surface β Δ B75KN-CTD protein	This study
MG1363 + pOri23. <i>bac</i> Δ FHBD	pOri23. <i>bac</i> Δ FHBD	MG1363 expressing surface β Δ FHBD protein	This study

Table 4.1. Bacterial strains used in Chapter 4.

4.2.2 Vector construction

The wild-type pOri23.*bac* vector (**Table 4.2**) was used as the template for inverse PCR to construct: (1) pOri23.*bac* Δ B75KN, a vector encoding β protein with an in-frame deletion of the B75KN domain (residues 504–788), using primers specific to the IgI3 (5'-GATCCGCGCCGCTTGTTCCTCTTCTTTGTC-3') and XPZ (5'-GATCCGCGCCGCGACGCCAGAACTCCAGATACACCG-3') domains and containing NotI restriction sites; and (2) pOri23.*bac* Δ FHBD, a complement factor H-binding QHLQKKN loop (FHBD; residues 223-QHLQKKN-229 to GAPG) using primers specific to the upstream (5'-AAGGTTTTAGATGGAGTTCATGGCGCGCCGGGCGATC-3') and

downstream (5'-GATCGCCCGGCGGCCATGAACTCCATCTAAAACCTT-3') sequence of the FHBD and containing *Ascl* restriction sites (**Table 4.3**). Standard protocol reaction mixtures of Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) were used. In brief, the following mixture was made: 12.5 μ L Mastermix + 9.5 μ L dH₂O + 1 μ L F primer (at 20 mM) + 1 μ L R primer (at 20 mM) + 1 μ L of template DNA (at 5 ng/ μ L). PCR was performed by thermocycling conditions of 2 mins at 98°C, 25 cycles of 15 s at 98°C, 30 s at 62°C, and 5 mins at 72°C, followed by a final cycle of 5 mins at 72°C. A small volume (4 μ L) of the PCR product was run on a 1% agarose gel to confirm successful amplification. Subsequently, the PCR product was digested with *DpnI* (Promega), followed by *NotI* digestion, and then ligated into *NotI* linearized pOri23 vector following manufacturer's protocols. The vectors were then transformed into *E. coli* DH5 α cells. Transformants carrying pOri23.*bac* vectors were selected by growth on LB + 500 μ g/mL erythromycin agar plates and individual colonies were screened by colony PCR using DreamTaq Green PCR Master Mix 2 $\times$ (Thermo Fisher Scientific). In brief, the following mixture was made: 12.5 μ L Mastermix + 9.5 μ L dH₂O + 1 μ L F primer (at 20 mM) + 1 μ L R primer (at 20 mM) + 1 μ L of template (*an E. coli* colony resuspended in 20 μ L dH₂O and boiled for 5 mins). PCR was set to 95°C for 5 mins, 35 cycles of 95°C for 15 secs, 60°C for 30 secs, 72°C for 60 secs, followed by 72°C for 10 minutes.

Vector	Notes	Reference
For surface expression of β proteins in <i>L. lactis</i>		
pOri23. <i>bac</i>	pOri23 carrying <i>bac</i> gene under control of p23 promoter	This study
pOri23. <i>bac</i> Δ B6C	pOri23 carrying <i>bac</i> Δ B6C insert under control of p23 promoter	This study
pOri23. <i>bac</i> Δ B75KN	pOri23 carrying <i>bac</i> Δ B75KN-CTD insert under control of p23 promoter	This study
pOri23. <i>bac</i> Δ FHBD	pOri23 carrying <i>bac</i> Δ FHBD insert under control of p23 promoter	This study

Table 4.2. Vectors used in chapter 4.

Oligonucleotide	Sequence (5' - 3')
<i>To produce A909 bac gene with FHBD domain deletion</i>	
<i>FHBD-F</i>	AAGGTTTTAGATGGAGTTCATGGCGCGCCGGGCGATC
<i>FHBD-R</i>	GATCGCCCGGCGGCCATGAACTCCATCTAAAACCTT

Table 4.3. Primers used in chapter 4.
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4.2.3 Expression and purification of recombinant β protein

Protein expression

Proteins used for mass spectrometry and crystallography analysis were performed by the Geisbrect lab.

4.2.4 Biochemical assays

Limiting proteolytic digestion of B75KN and mass spectrometry characterisation

Limiting proteolytic digestion of B75KN and mass spectrometry were performed by the Geisbrect lab.

Surface plasmon resonance analysis of β protein interactions with FH

Surface plasmon resonance assays were performed by the Geisbrect lab.

Protein structure determination by x-ray crystallography and structural analysis

X-ray crystallography was performed by the Geisbrecht lab.

Confirming B75KN and FH interactions by cross-linking and mass spectrometry

High-mass MALDI analysis and peptide mass fingerprinting were performed by the Geisbrecht lab.

SPR measurements of C3 convertase formation and decay

SPR C3 convertase formation and decay assays performed by the Geisbrecht lab.

4.2.5 Producing a β heterologous expression model using *L. lactis*

Competent *L. lactis* was produced by subculturing *L. lactis* in GM17 media (M17 media, 0.5% [w/v] glucose, and 0.5% [w/v] lactose) supplemented with 0.5 M sucrose at 30°C until mid-logarithmic phase ($OD_{600} = 0.7$). *L. lactis* was then incubated at 4°C for 10 min, centrifuged at 3,273 *g* for 15 min, washed three times by resuspending in 3 ml electroporation Buffer (0.5 M sucrose and 10% [v/v] glycerol), centrifuged at 2,250 *g* for 15 min, and resuspended in 100 μ L electroporation buffer. 100 μ L of competent *L. lactis* was transformed with 5 μ L pOri23 vectors by electroporation using a 1-mm gap electroporation cuvette at 1.2 kV and recovered by growth in 900 μ L M17 medium supplemented with 0.5 M sucrose, 0.5% (w/v) glucose, 20 mM $MgCl_2$, and 2 mM $CaCl_2$ on ice for 10 minutes followed by incubation at 30°C for 2 h. Transformants were selected by growth at 30°C on GM17 agar plates supplemented with 5 μ g/ml erythromycin after 48 hours.

Total β protein expression of bacteria was measured by SDS-PAGE analysis of cellular lysates. 1ml of bacteria cultured overnight was pelleted by centrifugation, then resuspended in 100 μ L sample buffer, and lysed by heating to 95°C for 10 min. Samples were separated on a 12.5% SDS-PAGE gel, washed in double-distilled H_2O , stained with Biosafe Coomassie Blue stain (Bio-Rad Laboratories), and destained with double-distilled H_2O .

Bacterial surface expression of β protein was measured by flow cytometry. Mid-logarithmic phase bacteria were incubated for 45 mins with either 0.1% (v/v) rabbit anti- β serum or normal rabbit serum (Lindahl et al. 1990), washed in PBS plus 0.5% (w/v) BSA, incubated for 45 mins with a 1:1,000 dilution of FITC-conjugated goat anti-rabbit IgG (Life Technologies), washed in PBS plus 0.5% (w/v) BSA, and fixed in 1% (w/v) paraformaldehyde (PFA). Fluorescence of bacteria was measured by flow cytometry analysis (FACS Calibur). Rabbit anti- β serum was generated as previously described (Lindahl et al., 1990) and supplied by Gunnar Lindahl (Lund University).

4.2.6 Bacterial complement assays

Measuring FH, C3b, and iC3b deposition on L. lactis by flow cytometry

FH binding phenotype of *L. lactis* strains was measured by flow cytometry. FITC-labelled FH (FH-FITC) was prepared as previously described (Haapasalo et al. 2015). Mid-logarithmic growth phase bacteria was incubated at room temperature for 45 mins with either 5 μ g/ml FH-FITC or buffer, washed in PBS plus 0.5% (w/v) BSA, and fixed in 1% (w/v) PFA. *L. lactis* fluorescence was measured by flow cytometry analysis (FACS Calibur).

C3b deposition on *L. lactis* was measured by flow cytometry. Stationary phase *L. lactis* was incubated at 37°C for 30 mins with either 3% (v/v) pooled active human serum (AS) or pooled heat-inactivated human serum (HIS), washed with 100 μ L PBS, then stained with a 1:2,000 dilution of goat anti-C3b-FITC polyclonal Ab (pAb; MP

Biomedicals) at 4°C for 60 min, washed by PBS, and finally fixed in 1% (w/v) PFA. *L. lactis* fluorescence was measured by flow cytometry. To measure levels of iC3b deposition the same assay was used but with different antibodies. *L. lactis* was strained with 5 µg/ml mouse anti-iC3b-neoantigen-IgG mAb (Bio-Rad Laboratories), 5 µg/ml mouse isotype control (eBioscience), or goat anti-C3b-FITC (MP Biomedicals), followed by staining with PE-conjugated goat anti-mouse IgG (1:1,000; Life Technologies) for detection of mouse Abs.

Measurements of C3b deposition on L. lactis by Western blot

Stationary phase *L. lactis* strains were incubated with 30% (v/v) AS for indicated time periods. *L. lactis* was rigorously washed five times by addition of 100 µl PBS, centrifugation at 2,250 *g* for 5 min, and discarding the supernatant. *L. lactis* pellets were resuspended in sample buffer, boiled at 95°C for 5 mins, and separated by SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane, blocked in 5% (w/v) non-fat dry milk, washed in TBST buffer (20 mM Tris [pH 7.4], 150 mM NaCl, and 0.1% [v/v] Tween-20), and stained with a 1:10,000 dilution of goat anti-C3 pAb (Complement Technology) at 37°C for 1 h. The membrane was then washed in TBST buffer, stained with a 1:10,000 dilution of HRP-conjugated donkey anti-goat IgG (The Jackson Laboratory) at 37°C for 1 h, and washed with TBST buffer. Chemiluminescent signals were detected using ECL Western blot substrate (Promega).

Analysis of FH cofactor activity for FI-mediated degradation of C3b

The purity of C3b, FI, and FH (Complement Technology) was measured by separation on a 12.5% SDS-PAGE gel under nonreducing conditions. 250 µl stationary phase bacteria at absorbance of OD₆₀₀ = 2.0 were pelleted in a 1.5 ml Eppendorf tube and incubated at 37°C for 60 mins in either 100 µl HEPES-buffered saline with calcium and magnesium chloride and with BSA (20 mM HEPES [pH 7.4], 140 mM NaCl, 5 mM CaCl₂, 2.5 mM MgCl₂, and 0.5% [w/v] BSA; HBS⁺⁺⁺) buffer or HBS⁺⁺⁺ buffer supplemented with 10 µg/ml purified FH. *L. lactis* was washed five times by addition of 1 ml HBS⁺⁺⁺ buffer, followed by centrifugation at 2,250 *g* for 5 min, and discarding of the supernatant. *L. lactis* was transferred to sterile 1.5 ml Eppendorf tubes after each washing step. After washing, *L. lactis* samples were resuspended in 50 µl HBS⁺⁺⁺ containing 10 µg/ml C3b and 10 µg/ml FI and incubated at 37°C for 30 min, washed and pelleted by centrifugation at 2,250 *g* for 5 min. The supernatant was transferred to a sterile 1.5 ml Eppendorf tube. For control samples, 50 µl HBS⁺⁺⁺ containing 10 µg/ml C3b and 10 µg/ml FI supplemented with or without 10 µg/ml FH was incubated at 37°C for 30 min. Samples were mixed with sample buffer containing 2-mercaptoethanol and processed for anti-C3 immunoblot as described above. Densitometry analysis was performed using the open-source image processing package Fiji to determine the mean density of the β band and α'45 fragment band.

4.2.7 Human serum and *ex vivo* human blood assays in *L. lactis*

Ethical approval and consent for use of human blood

Ethical approval for draw and use of human blood was obtained from the Regional Ethics Committee and Imperial College Healthcare NHS Trust Tissue Bank (Regional Ethics Committee approval no. 17/WA/0161, Imperial College Healthcare Tissue Bank Human Tissue Authority license no. 12275, and Imperial College Research Ethics Committee no. 19IC5166).

Serum survival assays

L. lactis resistance to complement-mediated immune responses was assessed by measuring the survival of *L. lactis* following incubation with human serum. 50 μ l mid-logarithmic phase bacteria (resuspended to 1×10^6 CFU/ml in HEPES-buffered saline with calcium and magnesium chloride [20 mM HEPES (pH 7.4), 140 mM NaCl, 5 mM CaCl_2 , and 2.5 mM MgCl_2]; HBS⁺⁺ buffer) and 85 μ l HBS⁺⁺ were combined with either 15 μ l 100% pooled active serum (AS) or 100% heat-inactivated serum (HIS; heated to 56°C for 30 mins) and incubated at 37°C. Samples were collected at designated time points, serially diluted in PBS, and plated onto GM17 agar plates, and cultured at 30°C overnight. CFU counts were taken the next day, and AS samples were normalised against HIS samples.

Neutrophil phagocytosis assays

Resistance of *L. lactis* strains to phagocytosis by neutrophils were measured by flow cytometry. Neutrophils were isolated from human blood and assay was performed as previously described (Zhao et al. 2020). FITC-labelled *L. lactis* strains were generated by incubating stationary phase bacteria with 1 mg/ml FITC (Sigma-Aldrich) for 30 min. Bacteria were washed five times with PBS and incubated in 3% (v/v) AS or HIS at 37°C for 30 min. Neutrophils were incubated with FITC-labelled complement-opsonised *L. lactis* at a multiplicity of infection (MOI) of 10 at 37°C for 30 mins with shaking, washed with PBS, and fixed in 1% (w/v) PFA. Extracellular bacteria were quenched by addition of 250 μ g/ml trypan blue dissolved in ice-cold 0.1 M citrate buffer (pH 4). Neutrophil fluorescence was then measured by flow cytometry. The results of each assay were expressed as the geometric mean normalised to a control group.

Whole blood killing assays

Survival of *L. lactis* strains was assessed in whole human blood. The inoculum concentration was determined by assays (not shown) optimising for observable differences in *L. lactis* killing between strains and/or conditions in whole blood after 30 mins incubation. 150 µl whole blood and 50 µl bacteria (mid-logarithmic phase bacteria resuspended to 1×10^7 CFU/ml in HBS⁺⁺ buffer) were mixed in a 1.5 ml Eppendorf tube and incubated at 37°C with rotation for 30 mins. Samples were taken and serially diluted into sterile PBS, plated onto GM17 agar plates, and cultured at 30°C overnight. CFU counts were measured and AS samples were normalised to HIS samples.

4.3 Results

4.3.1 The B75KN domain of β contains a FH binding motif

To investigate the functional consequences of FH interaction with β protein, the FH binding interface of β protein was first characterised. The β protein is a large 132kDa protein with numerous distinct structures that could contain potential FH binding interfaces. To narrow down the search, the domains within the β protein that bound to FH were identified.

A panel of β protein fragments that contained different domains including rB6C-IgI3-B75KN (residues 226-789), rB6C (residues 226-391), and rB75KN (residues 504-789), the location and sizes of which were based on previous literature (van Sorge et al. 2021) were expressed. Purified β protein fragments were injected over immobilised FH and the K_d was measured (**Fig 4.2B-D**). FH bound to rB6C-IgI3-B75KN, with a high affinity of $342 \text{ nM} \pm 10$, consistent with previous reports (Areschoug et al. 2002). Next, rB6C and rB75KN fragments were injected over immobilised FH (**Fig 4.2C-D**) showing that only rB75KN retained the ability to bind FH with a binding affinity of $595 \text{ nM} \pm 8.7$. Furthermore, these results were recapitulated using high-mass MALDI-TOF (**Fig 4.2E-G**). The formation of a single higher peak at 195,218 kDa after pre-treating rB75KN and FH with a bifunctional reactive amine linker (DSS) and mixing samples was observed, suggesting binding of rB75KN and FH (**Fig 4.2G**). To confirm that this peak was due to specific binding, and not due to addition of DSS alone, rB75KN (36.164 kDa, **Fig 4.2E**) or FH (162.039 kDa, **Fig 4.2F**) were DSS treated and peaks measured, which were similar to expected values and previous reports (Fenaille et al. 2007), demonstrating that DSS treated samples were acting as monomers. These findings unequivocally demonstrated that FH interacts with the B75KN domain of the β protein.

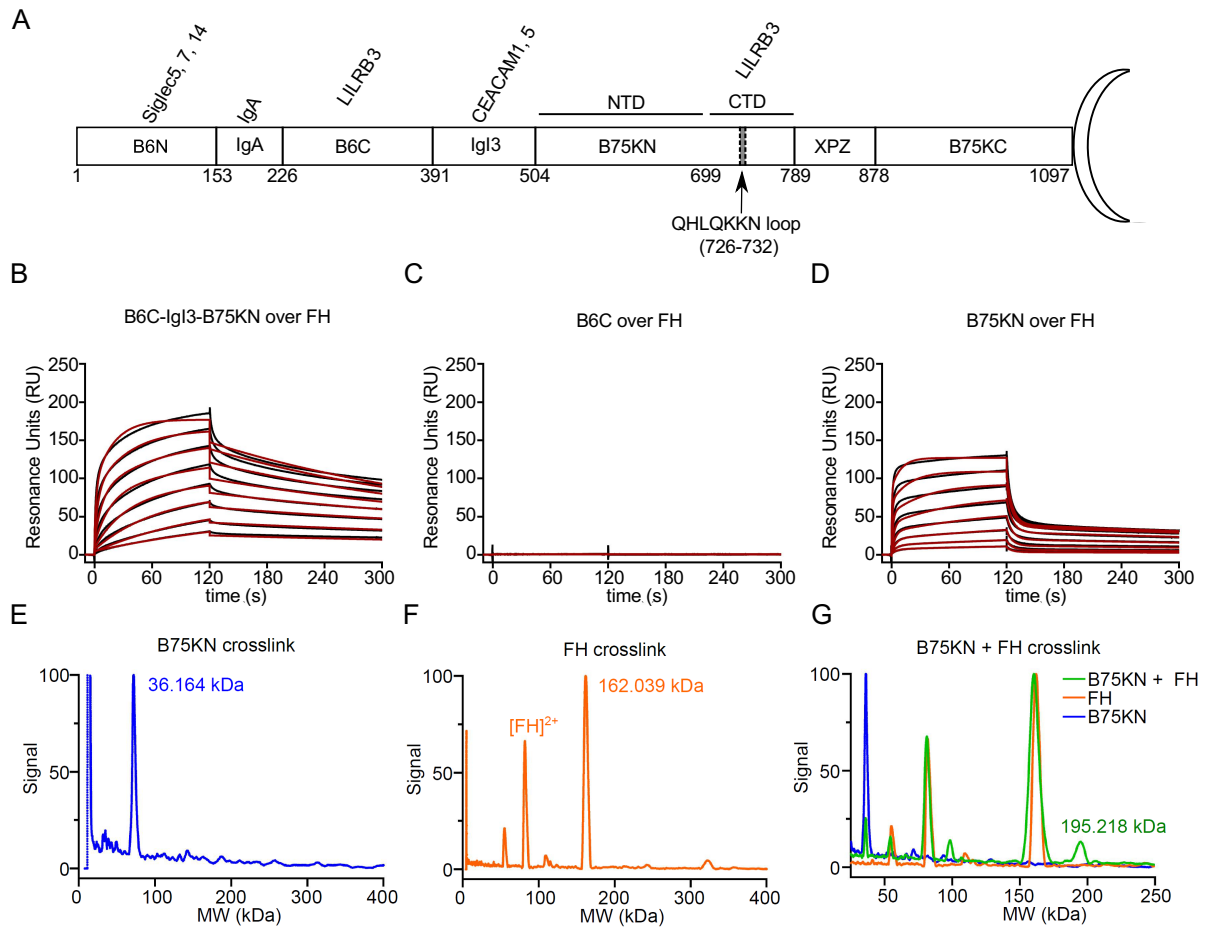


Figure 4.2. B75KN of the β protein contains a factor H (FH) binding motif. (A) Schematic representation of the β protein with location of key domains. The arrow indicates the location of the putative FH-binding loop (QHLQKKN loop) in the C terminal domain of B75KN. The binding domains of other human ligands of β protein are shown. (B-D) Reference-corrected SPR series for binding of the rB6C-IgI3-B75KN (B), rB6C (C), and rB75KN (D) fragments to immobilised FH, showing both the experimental data (black traces) and the fit to a ligand heterogeneity model (red traces). (E-G) High-mass MALDI-TOF spectra of a sample of rB75KN (E), FH (F), and cross-linked rB75KN and FH (G) following treatment with the bifunctional amine-reactive cross-linker DSS. rB75KN (blue), FH (orange), double charged FH, and rB75KN+FH cross-linked (green) peaks are labelled.

4.3.2 A disordered loop in B75KN-CTD (FHBD) is essential for FH binding

B75KN is a large domain with over >280 residues that make identifying regions of FH binding challenging. To localise the FH binding region of B75KN, we fragmented rB75KN into N and C terminal domains using proteolytic cleavage. rB75KN treatment using trypsin or subtilisin resulted in two stable domains: N-terminal domain (residues 504-687, rB75KN-NTD) and C-terminal domain (residues 688-789 rB75KN-CTD) (Fig 4.3A). The FH

interaction with both proteins was tested by injection of rB75KN-NTD and rB75KN-CTD over immobilised FH (**Fig 4.3B-C**). Only rB75KN-CTD retained the ability to interact with FH with a binding affinity of $864 \text{ nM} \pm 3.0$. Therefore, this indicated that FH binds to the B75KN-CTD region.

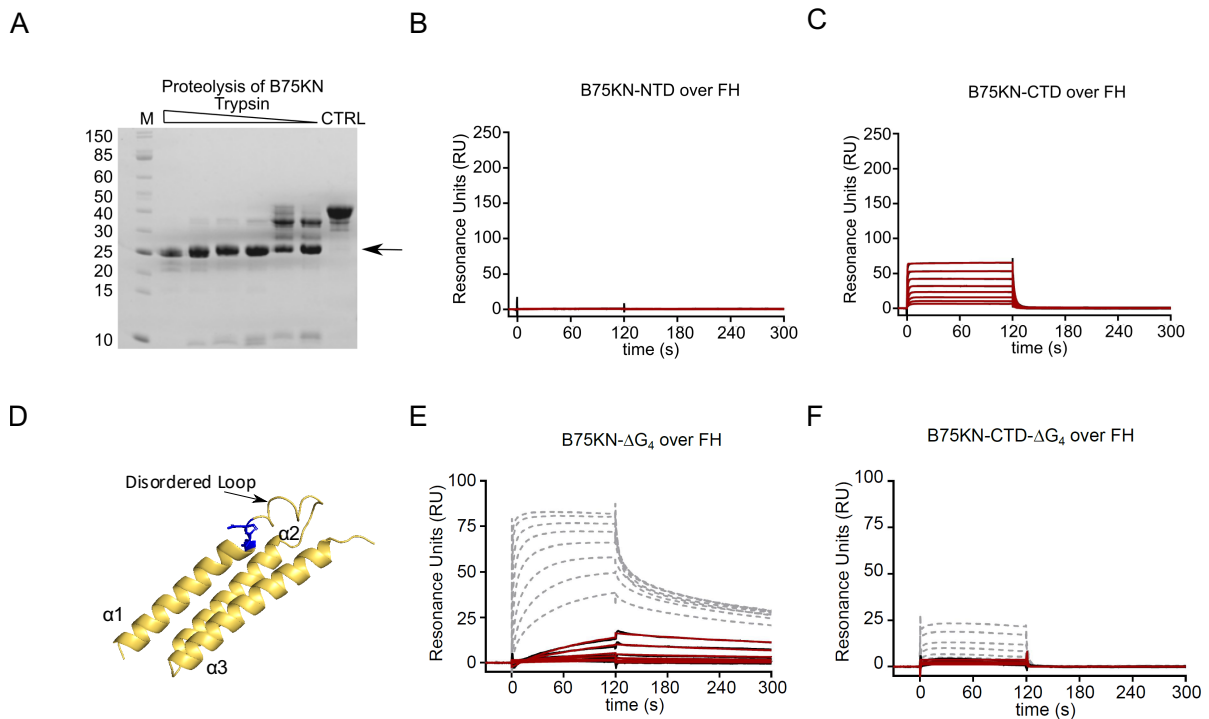


Figure 4.3. FH binds a disordered loop in B75KN-CTD (A) Limiting proteolysis of rB75KN by trypsin. SDS-PAGE analysis shows rB75KN fragment (CTRL) after incubation with increasing concentrations of trypsin for 30 min. Molecular size standards (M) are shown on the left of the gel image. Polypeptide species at ~25 kDa, indicated by an arrow, was sent for tryptic-peptide mass fingerprinting. (B-C) Reference-corrected SPR series for binding of the rB75KN-NTD (B) and rB75KN-CTD (C) to immobilised FH, showing both the experimental data (black traces) and the fit to a ligand heterogeneity model (red traces). (D) Representation of the B75KN-CTD structure determined by x-ray crystallography with LILRB3 interacting residues (blue) and the locations of the α helices and disordered loop labelled. (E-F) Reference-corrected SPR series for binding of the rB75KN- ΔG_4 mutant (E) and rB75KN-CTD- ΔG_4 mutant (F) to immobilised FH, showing both the experimental data (black traces) and the fit to a ligand heterogeneity model (red traces). An equivalent reference-corrected SPR series for binding of wild-type rB75KN is shown for comparison (dotted grey traces). SPR assays were performed in two independent experiments.

Having established that FH binds to B75KN-CTD domain of ~150 residues, the crystal structure of the rB75KN-CTD domain was solved by the Geisbrecht laboratory to understand the structural requirements for FH binding. The crystal structure of rB75KN-CTD was solved to 2.5 Å limiting resolution by utilising a seleno-L-methionine-labelled form of rB75KN-CTD (**Fig 4.3D**). The entire rB75KN-CTD domain (residues 699-787) was modelled except for residues 727-731, which had weak electron density, thereby, precluding it from being modelled. The crystal structure of rB75KN-CTD is defined by a 3 α -helix bundle fold. The 3 α -helices fold back on each other in an

antiparallel arrangement, with the hydrophobic side chains at the interface between these helices being highly complementary. This complementarity acts as a molecular zipper, holding the helices together.

There were no obvious grooves or obvious electrostatic features that suggested obvious candidate residues/regions that may bind FH. Therefore, the focus was directed at the disordered loop, residues 726-732, located between $\alpha 1$ and $\alpha 2$ loops (**Fig 4.2D**). Whilst there was no model for this region, the loop was thought to potentially interact with FH because the loop sequence – QHLKQKKN - contained several hydrogen bond donors/acceptors that may facilitate protein-protein interactions. Further, the low electron density of this disordered loop suggested it was highly dynamic in the absence of a binding partner.

To test whether QHLKQKKN was necessary for the FH interaction, site-directed mutagenesis of the B75KN domain was performed. We generated rB75KN and rB75KN-CTD proteins with the QHLKQKKN sequence replaced with a tetra-glycine linker: rB75KN- ΔG_4 and rB75KN-CTD- ΔG_4 , respectively. We then compared FH binding of wildtype rB75KN and rB75KN-CTD with our tetra-glycine mutants using surface plasmon resonance imaging (**Fig 4.3E-F**). rB75KN- ΔG_4 had significantly reduced ability to bind FH compared with wildtype (**Fig 4.3E-F**), whilst rB75KN-CTD- ΔG_4 binding to FH was abrogated (**Fig 4.3E-F**). These data strongly implied that the disordered loop (QHLKQKKN) is indeed a FH binding domain (FHBD).

4.3.3 Surface expression of β protein is sufficient to confer FH binding to *L. lactis*

Next, whether FH bound to β protein maintained functional activity, thereby conferring a potential immune evasion phenotype, was investigated. Previous studies had shown that surface expressed β protein adsorbs FH to the GBS cell wall from human serum (Areschoug et al. 2002); however, whether FH bound to β protein maintains functional activity is not understood. Determining the functional consequences of FH recruitment by β protein in GBS strains in the previous study was hampered by the fact that GBS also express Sht and ShtII that sequester FH at the surface (Maruvada, Prasadarao, and Rubens 2009; Moulin et al. 2019).

To ensure the functional characterisation of β protein and FH interactions in immune evasion was not confounded by other FH binding proteins expressed by *S. agalactiae*, a *L. lactis* heterologous expression system was used, as it does not bind FH. To specifically interrogate the role of FH-sequestered by β protein, *L. lactis* strains that expressed wildtype β protein (β), β protein with the B75KN domain knocked out ($\beta\Delta B75KN$), and β protein with the FHBD domain knocked out ($\beta\Delta FHBD$) were produced. First, the *bac* gene of GBS strains A909 was cloned into the pOri23 *E. coli*-*L. lactis* shuttle vector (Que et al. 2000) and subsequently expressed in *L. lactis* as revealed by SDS-PAGE analysis (**Fig 4.4A**), and as described in Chapter 3. Using the constructed β -expressing vector (pOri23.*bac*) as a template, pOri23.*bac* derivative vectors were generated by site-directed mutagenesis with either the entire B75KN domain or the FHBD loop mutated. To generate a vector for expression of $\beta\Delta B75KN$,

inverse PCR of pOri23.*bac* was performed using primers designed against B75KN-flanking domains Igl3 and XPZ to create an in-frame deletion of the B75KN domain. To generate a vector for expression of β protein with mutation of the QHLQKKN loop, inverse PCR of pOri23.*bac* was performed using primers designed upstream and downstream of FHBD that generated a QHLQKKN->GAPG. Comparison of wildtype and mutant β -expressing strains by SDS-PAGE analysis revealed that the *L. lactis* strains carrying pOri23.*bac* Δ B75KN and pOri23.*bac* Δ FHBD expressed β protein forms at slightly lower molecular weights, as expected (**Fig 4.4A**).

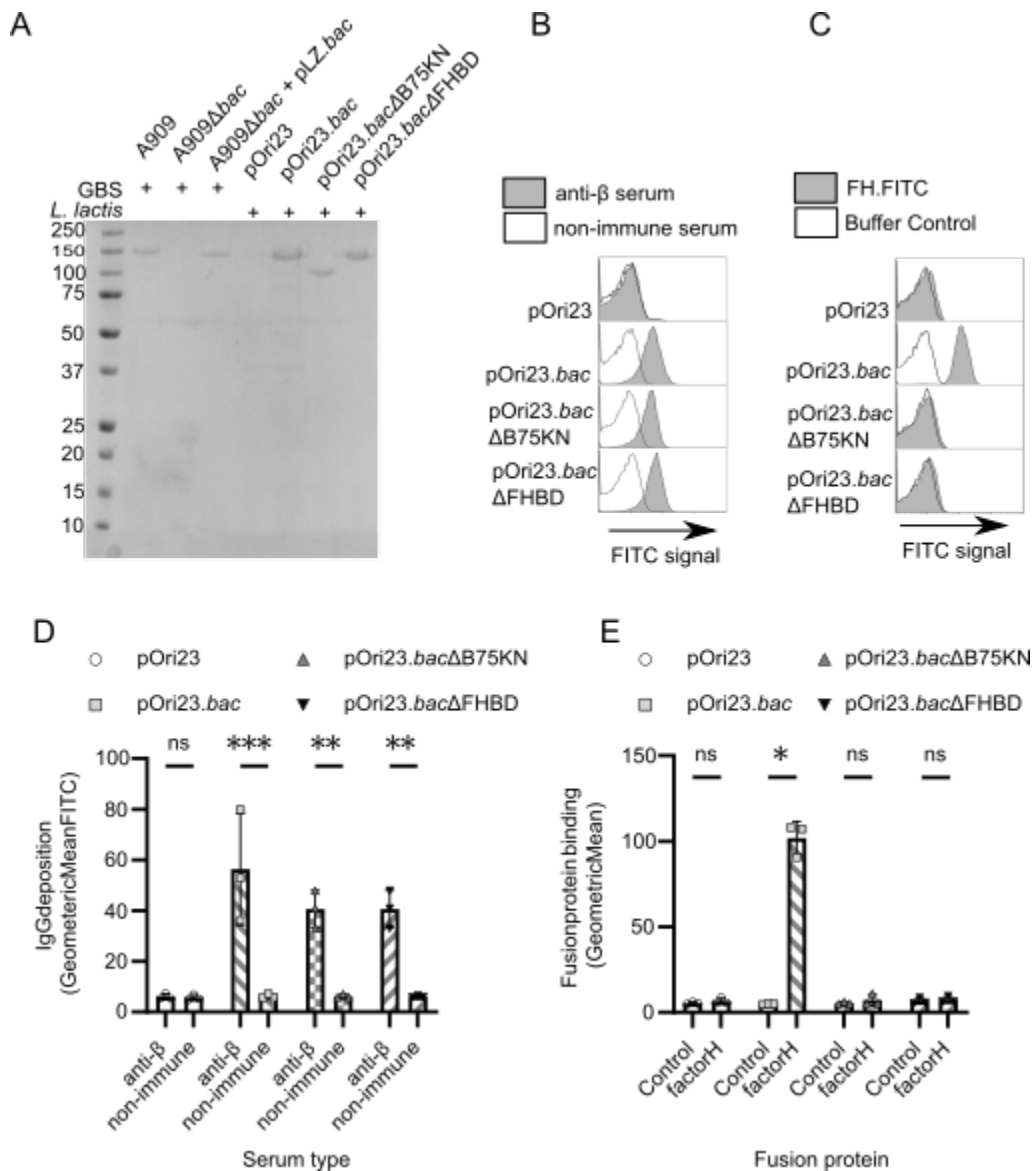


Figure 4.4. Surface expression of β protein is sufficient to confer FH binding *L. lactis*. (A) Expression of β protein by *L. lactis* strains, as measured by SDS-PAGE analysis. Lysates of stationary phase isogenic GBS strains or *L. lactis* strains carrying pOri23, pOri23.bac, pOri23.bacΔB75KN, or pOri23.bacΔFHBD vectors were assessed. (B, D) Surface expression of β protein of *L. lactis* strains, as detected by incubation in 0.1% (v/v) anti- β or non-immune rabbit serum and staining with anti-rabbit mAb (1:1,000), followed by flow cytometry analysis. Representative flow cytometry plot (B) and summary graph (D) with $n = 3$ replicates are shown. (C, E) Binding of FITC-labelled FH to the surface of *L. lactis* strains measured by flow cytometry. Representative flow cytometry plot (C) and summary graph (E) with $n = 3$ replicates are shown. Two-way ANOVA was performed to assess levels of statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

Having produced β -expressing *L. lactis* strains, the expression of β protein on the bacterial surface was assessed. To measure surface β expression, *L. lactis* strains were incubated with a rabbit anti- β serum and binding was

measured by flow cytometry (**Fig 4.4B, 4.4D**). Surface expression of β protein was confirmed across all β -expressing *L. lactis* strains, as shown by a representative flow plot (**Fig 4.4B**) and graph (**Fig 4.4D**). Importantly, both analyses indicated that all strains exhibited similar levels of β protein expression.

To assess whether *L. lactis* strains interacted with human FH as expected, each strain was tested for the capacity to bind FITC-labelled FH by flow cytometry, as shown by a representative flow plot (**Fig 4.4C**) and graph (**Fig 4.4E**). FITC-labelled FH bound wildtype β -expressing strain (*L. lactis* + pOri23.bac), indicating that β protein expressed on *L. lactis* can sequester FH on the bacterial surface, which is consistent with *S. agalactiae* strains (Areschoug et al. 2002). In contrast, FH was not sequestered by *L. lactis* strains expressing $\beta\Delta B75KN$ (*L. lactis* + pOri23.bac $\Delta B75KN$) or $\beta\Delta FHBD$ (*L. lactis* + pOri23.bac $\Delta FHBD$) (**Fig 4.4C, 4.4E**). These results showed that β protein sequesters FH and that this sequestration is dependent on the FHBD loop within the B75KN domain.

To conclude, *L. lactis* strains were produced that express surface wildtype, $\beta\Delta B75KN$ and $\beta\Delta FHBD$ proteins. Importantly, these strains bound FH as expected, and therefore, could be reliably used to interrogate the functional importance of the β -FH interaction regarding complement and immune evasion.

4.3.4 FH bound to β protein retains co-factor activity

Given that FH is a negative regulator of the complement system, it was hypothesised that FH bound to β protein retained its potent complement inhibitory phenotype. To test this, the co-factor activity of FH sequestered by β -expressing bacteria for FI-mediated inactivation of C3b to iC3b was measured. C3b, FI and FH were ordered from suppliers and initially tested for purity by SDS-PAGE, and the individual bands visualised demonstrated the purity (**Fig 4.5A**). The cofactor activity of β -bound FH was subsequently assessed using C3b, FI and FH in a widely used assay assessing FH function (**Fig 4.5A-B**). Briefly, *L. lactis* strains were incubated with purified FH (**Fig 4.5A**), extensively washed, and then incubated with purified C3b and FI. After removing the *L. lactis* by centrifugation, the supernatants were measured for breakdown of C3b to iC3b by Western blot analysis using an anti-C3 antibody (**Fig 4.5B**). In the absence of preincubation of *L. lactis* strains with FH, C3b was not degraded to detectable levels of iC3b (lanes 3-6, **Fig 4.5B**), indicating that *L. lactis* strains do not possess inherent membrane-anchored complement regulators, as expected. The *L. lactis* strain expressing wildtype β protein (pOri23.bac) degraded high levels of C3b to iC3b ($\alpha 45'$ band) in the presence, but not the absence, of FH (lanes 8, 6 **Fig 4.5B**). This degradation of C3b to iC3b was like that observed for the control when purified C3b, FI, and FH were incubated in the absence of bacteria (lane 2 **Fig 4.5B**). In contrast, *L. lactis* strains pOri23, pOri23.bac $\Delta B75KN$, or pOri23.bac $\Delta FHBD$ produced no detectable levels of iC3b after preincubation with FH (lanes 7,9,10 **Fig 4.5B**). Densitometry analysis of β band and $\alpha 45'$ band of four replicates, validated this result, and confirmed that levels of iC3b measured were not due to variation in total C3b (**Fig 4.5C**). A comparison of the α' band signal intensity between *L. lactis* + pOri23.bac samples indicated that 18% of C3b was degraded upon incubation with FH. In

comparison, 65% of the control C3b was degraded upon incubation with FH. These results showed that β -bound FH maintains its co-factor properties for FI-mediated inactivation of C3b to iC3b.

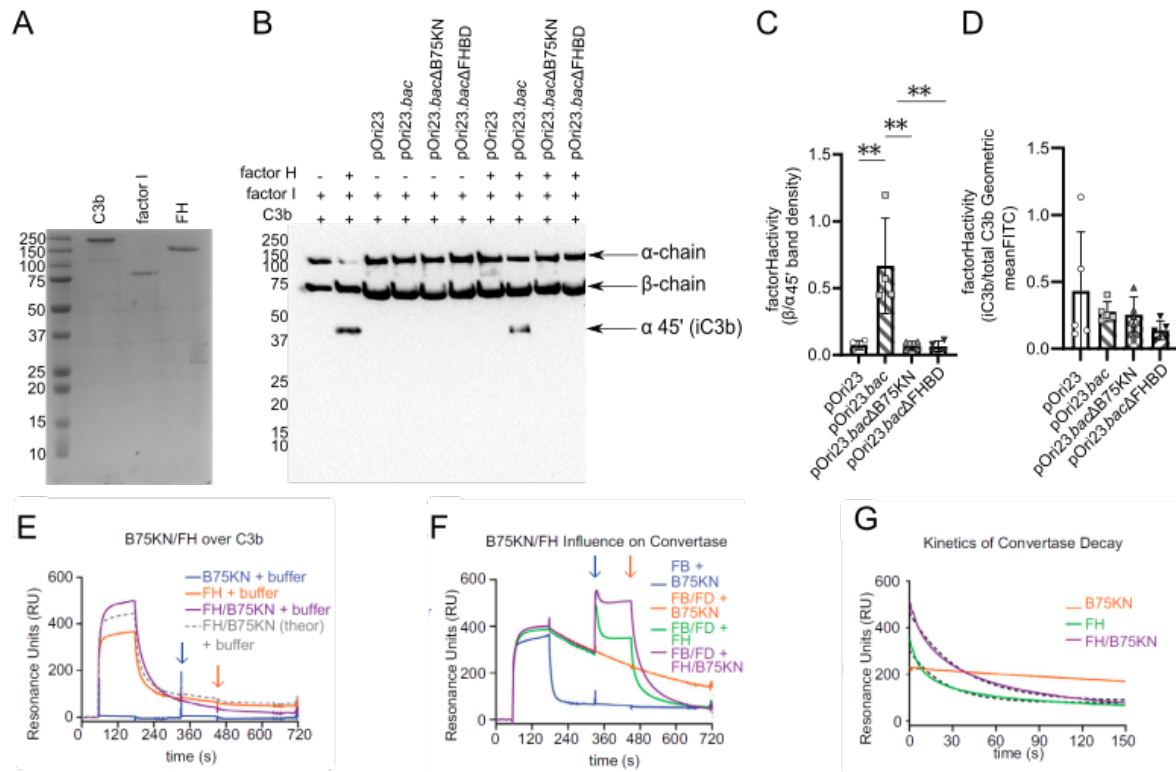


Figure 4.5. FH bound to β protein retains co-factor function. (A) Purity of C3b, FI, and FH, as measured by SDS-PAGE analysis. (B-C) C3b proteolytic co-factor activity of FH-bound to *L. lactis*, as measured by Western blot analysis. Strains were incubated with FH (10 μ g/ml) or buffer control, followed by addition of C3b (10 μ g/ml) and FI (10 μ g/ml) at 37°C for 30 min, then stained for total C3. Representative image (B) and summary graph of densitometry intensity (C) of $n = 4$ replicates are shown. (D) iC3b and C3b deposition on *L. lactis* strains after serum incubation measured by flow cytometry analysis. *L. lactis* strains were incubated with 3% (v/v) human serum, and stained for iC3b neoantigen (5 μ g/ml), mouse isotype control (5 μ g/ml), goat anti-C3b-FITC (1:2,000), or PBS control. The mean and SD of $n = 5$ replicates is shown. One-way ANOVA was performed to assess levels of statistical significance (** $p < 0.01$). (E-G) FH-B75KN binding to C3b and resultant formation/decay of C3bBb (C3 Convertase), as measured by SPR. FH-complexes were injected over a C3b coated biosensor and binding affinity measured. Representative sensorgrams show FH-C3b binding (E), formation of C3bBb convertase (F), and decay of C3bBb convertase (G). (E) Plot showing injection of 500 nM rB75KN (blue), FH (orange), rB75KN/FH complex (purple), or theoretic rB75KN/FH (grey dashed). (F) Plot showing injections of factor B (FB) followed by rB75KN (blue trace); and FB/FD mixture followed by rB75KN (orange trace), by FH (green trace), or rB75KN/FH complex (purple). The start (blue) and end (orange) of the second injection phase are marked by arrows. (G) Plot showing the rate of convertase decay for B75KN (orange trace), FH (green trace), or the rB75KN/FH complex (purple trace) following the second injection stop. Dashed black lines represent the data fit to a single exponential decay function. The experiments shown in (E-G) are representative of $n = 3$ replicates.

To understand the implications of enhanced FH-mediated C3b degradation exhibited by β -expressing bacteria, levels of iC3b on *L. lactis* surfaces after incubation with human serum was investigated. The hypothesis proposed was that FH-binding strains may have higher levels of iC3b deposited on the bacterial surface. *L. lactis* strains were incubated with 3% human serum and iC3b deposition was measured using a mAb against the neoantigen of iC3b and flow cytometry (**Fig 4.5D**). Interestingly, there was no significant differences in relative iC3b/C3b deposition between *L. lactis* strains, suggesting that whilst FH-bound to β -protein maintains co-factor activity for FI-mediated inactivation of C3b, the iC3b produced is released from the bacterial surface. Release of iC3b from the bacterial surface may be important for bacterial immune evasion because iC3b is recognised by complement receptor 3 (CR3) which is expressed by phagocytes such as neutrophils and macrophages (Newman, Becker, and Halme 1985).

Finally, whether FH bound to β protein maintained its additional complement regulatory activities to regulate C3bBb activity was determined. The decay of the C3bBb convertase was measured by injecting fixed concentrations (500nM) of rB75KN, FH, or FH/rB75KN over a C3b immobilised biosensor, as a control to understand the parameters of the SPR signal produced, i.e. the SPR magnitude and decay rate (**Fig 4.5E**). Furthermore, this revealed that rB75KN alone does not directly bind to C3b. Next, we injected a premixture of FB (blue) and FD (FB/FD) over C3b to produced C3bBb convertase, followed by either rB75KN (orange), FH (green) or rB75KN and FH in complex (purple, FH/rB75KN) (**Fig 4.5F**). Samples injected with FB/FD premixtures had much slower dissociation than FB alone (blue), suggesting that C3bBb has a slower rate of decay than FB to C3b alone. Consistent with B75KN not being able to directly bind C3b (**Fig 4.5E**), the rate of decay of C3bBb was unaffected by injection of rB75KN (orange). In contrast, when injecting FH over C3bBb (green), there was an increase in SPR signal, then equilibration of signal, and finally a rapid drop in SPR signal upon injection stop. This observation is consistent with FH acting to accelerate the decay of C3bBb convertase (**Fig 4.5 F-G**). When we injected FH in complex with rB75KN, we observed a similar SPR signal, consistent with a larger complex, showing rapid accelerated decay of C3bBb convertase (**Fig 4.5 F-G**). These results showed that FH bound to rB75KN retains its functional activity to accelerate the decay of C3bBb convertase.

These data showed that FH bound to β protein maintains its functional activity to inhibit/degrade key components of the alternative complement pathway, namely C3b and C3bBb convertase. FH bound to β protein maintains its ability to (1) act as a cofactor for FI to proteolytically inactive C3b to iC3b and (2) accelerate the decay of C3bBb convertase.

4.3.5 β expression reduces C3b deposition

Having shown that FH maintained its functional activity whilst bound to β protein, whether FH-sequestration by β protein modified complement C3 deposition on the bacterial surface was investigated. In brief, *L. lactis* strains were incubated in human serum for 5, 15, 30 and 60 minutes, and the β chain of C3b, and α 67' and α 45' chains

of iC3b were probed by Western blot (Fig 4.6A). The analysis showed the wildtype β -expressing strain had reduced C3b and iC3b deposited onto the *L. lactis* surface compared with all other strains at all timepoints measured (β band, Fig 4.6A).

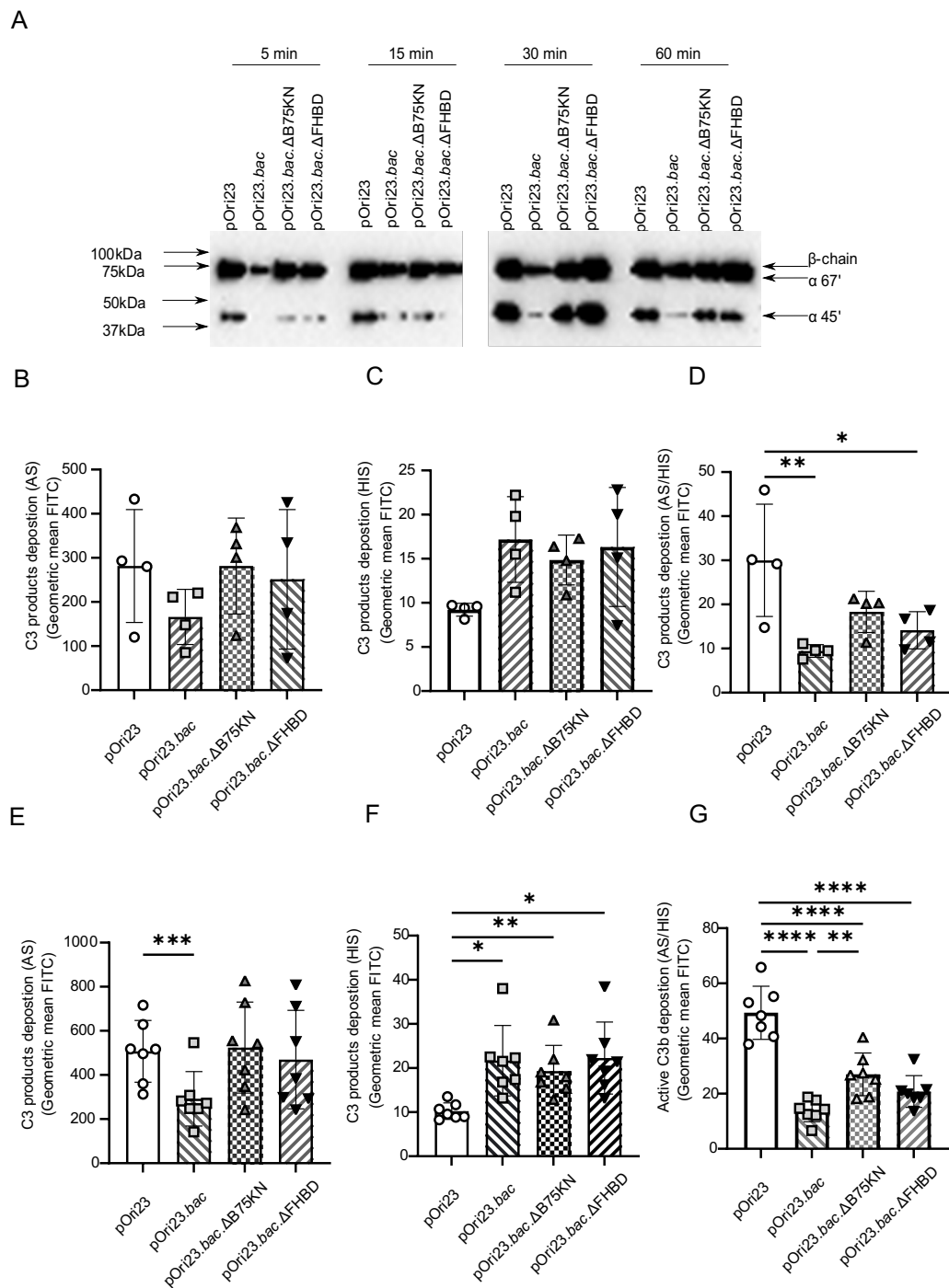


Figure 4.6. β expression reduces C3b deposition. (A) Western blot analysis showing C3b and iC3b deposition on stationary phase *L. lactis* strains after incubation at several timepoints with 30% (v/v) human serum. C3b and iC3b were stained using anti-C3-HRP pAb (1:10,000). Data are representative of $n = 2$ assays (B-G) Stationary phase *L. lactis* strains were incubated in 3% (v/v) AS or HIS at 37°C for 30 mins (B-D), or 60 mins (E-G) stained with goat anti-C3-FITC pAb. Total C3b deposition of AS relative to HIS was quantified. Mean and SD of $n= 4-7$ replicates are shown. One-way ANOVA was performed to determine statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; **** $p < 0.005$).

To further examine the phenotype, the total C3b deposition onto the surface of *L. lactis* was quantified using flow cytometry. In these assays, the C3b deposition onto *L. lactis* strains that had been incubated in active serum (AS, Fig 6B, E) or heat inactivated serum (HIS, Fig 6C, F) was measured, and the relative C3b deposition due to active serum was calculated (AS/HIS, Fig 6C, G). Heat inactivated serum was used to control for differences in C3b binding between strains and variation of C3b between human serum used. Expression of wildtype β protein on the surface of *L. lactis* led to a significant reduction in total C3b deposition compared with empty pOri23 vector control (Fig 6D) after 30 minutes. *L. lactis* strains expressing $\beta\Delta B75KN$ and $\beta\Delta FHBD$ had reduced C3b deposition compared with empty vector, and there was a trend for these strains to have greater C3b deposition compared with β wildtype, but it was not statistically significant. However, after 60 minutes of incubation, there was significantly less C3b deposition on β wildtype than $\beta\Delta B75KN$, demonstrating that the B75KN is important for C3b deposition. Whilst $\beta\Delta FHBD$ was not significantly different from β wildtype, there was a trend for enhanced C3b deposition on $\beta\Delta FHBD$ expressing strains, suggesting that the FHBD and therefore FH binding, is the main cause of the differences observed in C3b deposition between strains. In conclusion, *L. lactis* expressing β protein had reduced C3b deposition on its surface after incubation in human serum and this reduction in C3b deposition was impaired by the deletion of B75KN and FHBD domains. These data demonstrate that FH binding to β protein is responsible for reducing total C3b deposition.

4.3.6 Functional consequences of β -FH on immune evasion

It has been repeatedly reported that pathogen sequestration of FH and inhibition of C3b deposition is associated with enhanced capacity of pathogens to evade innate immune responses (Ferreira, Pangburn, and Cortés 2010). Therefore, if β protein enhanced resistance to complement-mediated killing and opsonophagocytic killing through FH sequestration was investigated. First, whether FH binding by β protein influenced serum-mediated killing of bacteria was tested. The number of surviving bacteria after 2 hours incubation in human active serum and heat-inactivated serum was measured by colony forming unit (CFU) quantification (Fig 4.7A). There was no difference in *L. lactis* survival after incubation in human active or heat-inactivated serum for all strains, indicating that expression of β protein did not modify resistance to complement-mediated killing nor modify bacterial growth in serum.

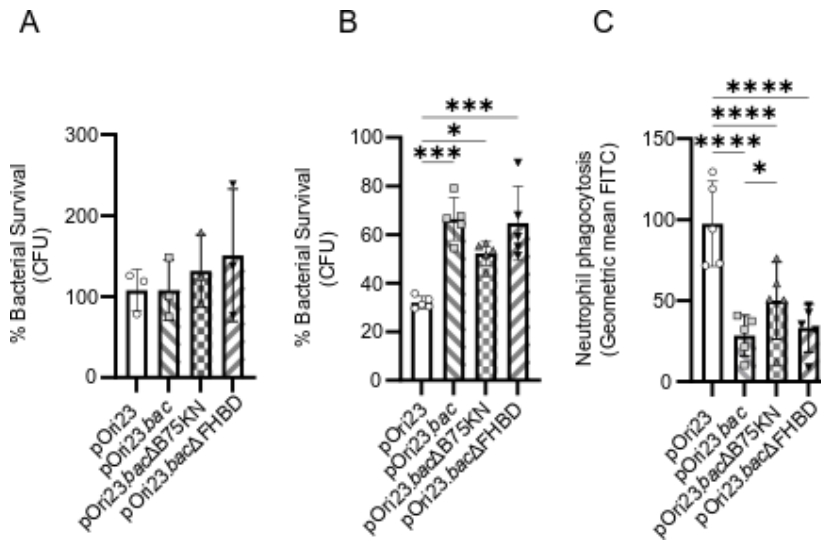


Figure 4.7. B75KN domain attenuates neutrophil phagocytosis and whole blood survival independently of FH binding. (A). Mid-logarithmic phase *L. lactis* strains were incubated with 10% (v/v) serum (AS) or heat-inactivated serum (HIS) at 37°C for 120 mins. The number of CFUs were measured and bacterial survival was determined by AS/HIS. (B) Mid-logarithmic phase *L. lactis* strains were inoculated into human whole blood and incubated at 37°C. CFU count was measured, and the percentage of bacterial survival was determined as in (A). (C) Phagocytosis of FITC-labelled *L. lactis* strains by human neutrophils after opsonisation in human serum. FITC-labelled stationary phase *L. lactis* strains were incubated with human neutrophils at a multiplicity of infection of 10 for 30 mins at 37°C with shaking. Neutrophils were washed and fluorescence measured by flow cytometry. Mean and SD of $n = 5$ replicates is shown. One-way ANOVA was performed to determine statistical significance (* $p < 0.05$; *** $p < 0.005$; **** $p < 0.001$).

Next, whether β protein and FH interactions enhance resistance to neutrophil-mediated opsonophagocytosis was tested. FITC-labelled *L. lactis* strains were opsonised with human active serum for 30 minutes, incubated with human neutrophils for 30 minutes, and the fluorescence of neutrophils was measured by flow cytometry (Fig 4.7C). The β -expressing *L. lactis* strain showed enhanced resistance to neutrophil opsonophagocytosis compared with the empty pOri23 vector control. Strains expressing $\beta\Delta B75KN$ had significantly attenuated resistance to neutrophil-mediated opsonophagocytosis compared with wildtype β ; however, $\beta\Delta FHBD$ exhibited similar resistance to wildtype β .

Finally, the effect of the β -FH interaction on whole blood killing was investigated. *L. lactis* strains were opsonised with human serum, then incubated with whole human blood, and the number of surviving bacteria was quantified by CFU count measurement (Fig 4.7B). β -expressing strains exhibited enhanced resistance to killing in whole blood compared with the *L. lactis* strain carrying the empty pOri23 vector. However, *L. lactis* strains carrying pOri23.bacΔB75KN, or pOri23.bacΔFHBD vectors exhibited similar levels of bacterial survival to the β

wildtype expressing strain. These results suggested FH binding to β protein is not necessary for β -mediated enhanced resistance to whole blood killing.

To conclude, these data showed that β protein expression is important for resistance to whole blood killing and neutrophil-mediated opsonophagocytosis. Furthermore, the data demonstrated that the B75KN domain is involved in promoting neutrophil-mediated opsonophagocytosis; however, this phenotype was independent of FH binding.

4.4 Discussion

There is a vast body of literature detailing the ingenious strategies and mechanisms pathogens employ to evade and/or suppress complement activation (Lambris, Ricklin, and Geisbrecht 2008). Of these strategies, none has been more widely studied than FH sequestration with ~100 FH-binding molecules being identified that are expressed by human pathogenic bacteria, viruses, protozoa, fungi and helminths (Moore et al. 2021). Furthermore, FH sequestered to microbial surfaces often retains its complement inhibitory function, with microbes utilising similar mechanisms to bind FH as host cells (Moore et al. 2021). Thus, pathogens that sequester FH inhibit complement activation on their cell surfaces. The fact that many human pathogens have independently evolved FH sequestration mechanisms suggests there must be some selective advantage for pathogens to sequester FH and suppress complement activation. The obvious question to ask is, *how* does suppression of complement provide this selective advantage? Due to the integral role of complement for innate immune activation there appears an obvious answer: pathogens suppress complement activation for immune evasion. However, evidence specifically linking FH-mediated complement suppression with bacterial immune evasion remains elusive, and there are conflicting *in vitro* and *in vivo* data on the importance of pathogen sequestration of FH for immune evasion (Woodman et al., 2007). Though *S. agalactiae* β protein has been shown to interact with FH, the mechanism of interaction and consequences for complement suppression and/or immune evasion are not well understood. This work has investigated the role of β -FH interaction for complement and immune evasion, and the findings contribute to addressing fundamental questions about bacterial biology and to providing information required to develop novel therapeutics to prevent or treat *S. agalactiae* infections.

In this study, the FH binding motif (QHLKQKKN) located in the B75KN-CTD fragment of β protein, that shares structural homology with other FH binding proteins, was identified. The crystal structure of the β fragment B75KN-CTD that contains the FHBD is composed of a three α -helix bundle with an up-down-up antiparallel topology. Interestingly, B75KN-CTD shares 27% structural homology to *S. pneumoniae* FH binding protein PspC3.4 (Chapter 5), suggesting a potential common structural FH binding motif expressed by streptococcal pathogens (Achila et al. 2015; Du et al. 2021). Whilst β protein and PspC may share a common FH binding motif, the functional consequences for complement inhibition appear to vary greatly. PspC binding to FH not only anchors FH to its surface but enhances the complement inhibitory activity of FH by exposing an additional C3b

binding epitope on the FH surface. FH in complex with PspC has been shown to exhibit a two-fold increase in C3b binding, and 5-fold increase in decay accelerating of C3bBb (Herbert et al. 2015). In contrast, there was no enhancement of C3b binding or decay accelerating rate of C3bBb by FH-bound to B75KN protein fragment, compared with FH buffer control. Instead, the data showed that β protein functions solely to bind FH with high affinity, acting as a molecular anchor for FH to the bacterial surface. These differences in complement regulation activity can be explained by the different CCP domains that β protein and PspC target. PspC binds CCP9, whilst cross-linking mass spectrometry analysis points to β protein binding to CCP3-4 (data not shown, Xu et al. 2022), which is perhaps unsurprising because CCP9 and CCP4 are the most similar based on structural alignment (Achila et al. 2015). As CCP1-4 contains the C3b binding interface, B75KN-CTD in complex with FH may be unable to expose further C3b epitopes required to enhance FH complement regulatory activity.

Heterologous expression of β protein in *L. lactis* demonstrated the remarkable ability of β protein expression alone to enhance bacterial immune evasion. However, the data has raised important questions about the role of sequestering FH for bacterial immune evasion. *L. lactis* expressing β protein lacking the FHBD exhibited the same resistance to neutrophil phagocytosis and had the same bacterial survival as strains expressing wildtype β protein. Contrary to the central dogma that bacteria sequester FH to inhibit complement which enhances immune evasion, the work presented suggests a more complex role played by sequestered FH. Whilst *S. agalactiae* may require expression of additional proteins in conjunction with β protein for FH-mediated immune evasion, the most likely explanation is that β protein enhances immune evasion through a FH-independent mechanism. This theory is supported by the knowledge that β protein interacts with inhibitory immune receptors LILRB3, CEACAM1, Siglec5 and Siglec7, and host immune-related factors such as IgA (Carlin et al. 2009; Fong et al. 2018; van Sorge et al. 2021). Indeed, *L. lactis* expressing β protein with the B75KN domain knocked out showed statistically significant reduction in neutrophil phagocytosis and reduced bacterial survival compared with strains expressing wildtype β protein. As previously discussed in Chapter 3, B75KN also binds LILRB3, whose ligation may suppress immune responses, thereby, enhancing immune evasion. Thus, the loss of LILRB3 interaction may be the cause of the observed differences in immune evasion between strains expressing wildtype β protein and $\beta\Delta$ B75KN protein.

Alternatively, the observed B75KN-mediated immune evasion may be the result of contributions from both LILRB3 and FH working synergistically to enhance immune evasion. This theory is supported by previous studies showing that LILRB3 variants may be involved in complement regulation through binding complement factors including C3b, iC3b, and C4b (Hofer et al. 2016). Thus, FH sequestered by β protein may enhance LILRB3 activation by producing C3b and iC3b that cross-link LILRB3, thereby triggering inhibitory signalling in neutrophils and suppressing the immune responses. In addition, LILRB3 and FH may act synergistically to negatively regulate FcR receptor activation and expression, thereby suppressing immune responses. FcR receptors are key mediators of neutrophil activation and effector functions such as opsonophagocytosis, degranulation and NETosis. LILRB3 ligation inhibits activating FcR receptors and suppresses neutrophil effector functions (Zhao et al. 2020), whilst FH activity reduces production of potent anaphylatoxin C5a, thereby downregulating expression of FcR on immune cell surfaces (Atkinson 2006). Thus, B75KN-mediated immune evasion may be due to FH and

LILRB3 suppressing neutrophil FcR expression and activity, respectively, thereby dampening neutrophil immune responses. Therefore, the β protein may exhibit redundancy for immune evasion mechanisms as a result of evolutionary pressure with β protein not relying on any single interaction to a ligand for overall immune evasion activity, and thus, ensuring the highest chance of survival, similar to the Sbi ligand of *S. aureus* (Haupt et al. 2008).

Another explanation for the apparent lack of involvement of FH in β -mediated immune evasion may be that the experimental conditions used may not have been optimal for measuring FH-mediated immune evasion by *S. agalactiae* β protein. The physiological environment and/or growth state are important factors influencing expression of FH binding proteins and FH recruitment to bacterial surfaces such as (1) *S. pneumoniae* expression of PspC at the division septum to recruit FH in growth phase (2) *S. agalactiae* upregulation of ShtI and ShtII expression only in zinc-restricted media (zinc being abundant in human serum) (3) Fba expression by *S. pyogenes* only binding FH during exponential phase of growth (Haapasalo et al. 2012; Pathak et al. 2018; Moulin et al. 2019). *L. lactis* in exponential phase of growth was tested using standard neutrophil and whole blood assays to measure immune evasion using flow cytometry and CFU counting; however, *L. lactis* may not bind FH efficiently under these physiological conditions. Using alternative media, growth states, and tools may increase FH binding to *L. lactis* and reveal FH-mediated immune evasion. Thus, β protein sequestration of FH for immune evasion may depend on the host environment and growth state of the bacterium.

These findings may also suggest a non-canonical role for bacteria to sequester FH. There is a large body of literature reporting bacterial pathogens sequester FH for immune evasion such as *S. pyogenes*, *S. pneumoniae*, *N. meningitidis*, and *B. burgdorferi* (Ermert, Ram, and Laabei 2019); however, many of these *in vitro* studies have not been supported *in vivo*. For example, *Borrelia* pathogens are model organisms to investigate FH-mediated immune evasion, due to expression of several FH binding proteins termed complement-regulator acquiring surface proteins (CRASP). Whilst *in vitro* studies into *Borrelia* claim FH sequestered by *Borrelia* enhances immune evasion (Kraiczky et al. 2003; Röttgerding et al. 2017), *in vivo* work showed that FH-deficient mice were no more susceptible to *Borrelia* infection compared with wildtype (Woodman et al. 2007). These conflicting reports between *in vitro* and *in vivo* studies may be due to FH species between human and mice sharing only ~60% sequence homology (Lu et al. 2008). If not for immune evasion, what function does sequestering FH play for bacteria? It has been proposed that bacteria sequester FH to enhance adhesion to host cells. Studies investigating *M. hypopneumoniae* and *S. pneumoniae* have shown that these bacteria utilise FH sequestration to enhance binding to epithelial cells (Hammerschmidt et al. 2007; Yu et al. 2020). Furthermore, *in vivo* studies of *S. pneumoniae* and *S. suis* suggest that enhanced adherence to epithelial cells contributes to invasion in mouse and pig models, respectively (Quin et al. 2007; Li et al. 2017). Therefore, β protein may sequester FH for a non-canonical role such as to enhance adhesion to host cells, thereby promoting invasion.

In conclusion, this work has shown that *S. agalactiae* expresses β protein for immune evasion, and that sequestered FH inhibits complement deposition on bacterial surfaces. However, the role FH plays for immune

evasion remains unclear. In future work, it would be interesting to investigate whether LILRB3 and FH work synergistically for immune evasion and to test different physiological environments/niches. Another direction of research that would be interesting to pursue is whether β protein sequesters FH for a non-canonical purpose, perhaps by promoting adhesion to epithelial cells. It would be interesting to characterise non-canonical roles for sequestering FH to enhance bacterial virulence and/or invasion, not just for *S. agalactiae*, but more broadly for other FH binding pathogens, and to solve the puzzle of why bacterial pathogens sequester FH.

Chapter 5. LILRB3 binds a subset of *S. pneumoniae* PspC proteins

5.1 Introduction

Streptococcus is a genus of Gram-positive bacteria containing more than 70 different species that can colonise and/or cause invasive diseases in humans and animals (Richards et al. 2014). They are classified into 6 major classes that are largely based on clinical features and reflects their phylogenetic relationships: *pyogenic*, *mitis*, *anginosus*, *salivarius*, *bovis* and *mutans* (Barajas et al. 2019; Yu et al. 2018). Certain classes of streptococci largely contain pathogenic species, such as the *pyogenic* (pus forming) class that contains *S. pyogenes*, in contrast to *S. mitis* that largely contains commensals (Sadowy et al. 2020). Whilst *Streptococcus* represents a diverse genus, analysis of over 100 strains from 16 *Streptococcus* species identified 404 proteins that were shared among all species (Barajas et al. 2019). Indeed, despite lateral gene transfer (LGT), certain biochemical processes have been retained within each class since formation; for example, carbohydrate metabolism by the *pyogenic* class (Richards et al. 2014).

Streptococcal species share virulence and immune evasion strategies. The vast majority of research has focused on characterising virulence factors expressed by *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae* due to their high global health burden (Yu et al. 2018). As a consequence, there is broad understanding of the commonalities and differences in virulence and immune evasion strategies between these pathogenic species. An early genomic study revealed that *S. agalactiae*, *S. pyogenes* and *S. pneumoniae* genomes shared 401 putative proteins (Yu et al. 2018). The increasing availability of genomes sequences of bacterial isolates over the last 15-20 years has provided datasets to allow more in-depth comparative genome analysis and opportunities to understand the similarities and differences between bacterial species. Indeed, the emergence of the pan-genome concept means that there is a large reservoir of genes present within the entire population of a bacterial species. Research has led to the identification of common virulence factors that are expressed by multiple species. For example, the R28 protein is a surface molecule expressed by *S. pyogenes* that is also expressed by several *S. agalactiae* strains, referred to as R28 or Alp3 (Stålhammar-Carlemalm et al. 1999). Another example is the haemolytic virulence factor streptolysin S (SLS) protein expressed by *S. pyogenes* which is homologous to cytolysin expressed by *S. iniae* (Fuller et al. 2002). Expression of protein homologs can result in common functional phenotypes and immune evasion strategies between different streptococcal species such as haemolysis of erythrocytes by SLS/cytolysin (Baiano and Barnes 2009).

Protein domains are shared between non-homologous proteins in streptococcal species that confer virulence phenotypes. The R28 protein expressed by *S. pyogenes* and β protein expressed by *S. agalactiae* both contain a IgI3 domain that enables binding to human immune receptor CEACAM1 (Catton et al. 2023; van Sorge et al. 2014). Binding of R28 to CEACAM1 has important phenotypic and biological consequences, with the interaction of R28 with CEACAM1 enhancing bacterial adhesion to epithelial cells and suppressing wound healing, thereby driving bacterial sepsis (Catton et al. 2023). Another example is the Siglec-like domain expressed by the sialic acid-binding adhesins GspA and SrpA expressed by *S. gordonii* and *S. sanguinis* species, respectively (Deng et al. 2014). The Siglec-like domain has a V-set Ig-like fold that enables binding to sialic residues expressed by platelets, which has been proposed as the mechanism that drives *S. gordonii* and *S. sanguinis* endocarditis (Deng et al. 2014). Thus, specific protein domains expressed by virulence factors can play a crucial role in determining disease pathogenesis.

The identification of LILRB3 binding domains (B75KN-CTD and B6C) in β protein of *S. agalactiae* provides an opportunity to identify structurally related virulence factors from different streptococcal species that could also interact with LILRB3. *S. agalactiae* β protein shares homology to the PspC proteins expressed by *S. pneumoniae* (Jarva et al. 2002). Work by the Meri group in 2002 showed that a subset of PspC variants, called Hic proteins and β protein both contained a homologous putative FH-binding domain that promoted evasion of the complement cascade (Jarva et al. 2002). Thus, *S. agalactiae* and *S. pneumoniae* express β and Hic proteins that contain a homologous domain that enables sequestering FH to presumably inactivate complement. Similarly, the IgI3 fold of β protein that interacts with CEACAM receptors has homologous domains in other streptococcal surface proteins such as *S. pyogenes* R28 protein (van Sorge et al. 2021). Given the knowledge that β protein has homologous domains in virulence factors from other bacterial species, it is now important to assess whether homologs of the LILRB3-interacting domains of β protein are found in virulence factors from *S. pneumoniae* and other streptococci. This is important for more broadly understanding of LILRB3 and bacteria interactions.

The work outlined in this chapter will focus on *S. pneumoniae*, an opportunistic pathogen that is a major global health burden. *S. pneumoniae* was estimated to cause 1.6 million deaths in 2005, of which 1 million were in children <5 years old (Dion and Ashurst 2023). Whilst *S. pneumoniae* generally acts as a commensal, being harmlessly carried by up to 27-65% of children in their upper respiratory tract, *S. pneumoniae* invasion into sterile environments such as the lungs, blood and meninges can lead to life-threatening diseases such as pneumoniae, sepsis, and meningitis (Weiser, Ferreira, and Paton 2018). There are ~100 *S. pneumoniae* serotypes that are based on the expression of a polysaccharide capsule, with specific serotypes 1, 4, 5, 7F, 8, 12F, 14, 18C and 19A associated with invasive disease (Song, Nahm, and Moseley 2013). Key virulence factors contribute to the remarkable ability of *S. pneumoniae* to evade the immune system and cause a range of diseases. The most well characterised virulence factors that contribute to immune evasion include the capsular polysaccharide, pneumolysin, and choline binding proteins, PspC and PspA (Brooks and Mias 2018). The choline binding proteins are surface molecules that directly interact with host factors such as FH and IgA, and their characterisation is key for understanding *S. pneumoniae* pathogenesis.

5.1.1 Hypothesis and aims

The hypothesis proposed was that structural homologs of the B75KN-CTD domain in β protein are present in other streptococcal surface proteins and these proteins interact with LILRB3. The aims were to (1) identify proteins expressed by non-*S. agalactiae* species that contain domains homologous to B75KN-CTD, (2) test a panel of recombinant proteins identified for LILRB3 binding, and (3) to determine the functional consequences for LILRB3 interaction with identified proteins.

5.2 Materials and methods

5.2.1 Bacterial strains and growth conditions

Bacterial growth conditions

E. coli strains (**Table 5.1**) were cultured in Lysogeny broth (LB, Invitrogen) or on LB agar plates (LB + 1.5% [w/v] bacterial agar VWR) at 37°C with shaking or statically, respectively. For strains containing pRSET-C, LB was supplemented with 100 μ g/mL ampicillin. *S. pneumoniae* (**Table 5.1**) was plated on Blood Agar plates (TH + HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (ThermoFisher) + 5% [v/v] horse defibrinated blood (ThermoFisher) + 1.5% bacterial agar [w/v] (VWR)), at 37°C with 5% CO₂. Starter cultures were prepared from frozen master stocks plated on Blood Agar plates and grown overnight at 37°C with 5% CO₂. Colonies were picked from overnight plates and cultured to mid-log phase in TH + HEPES, then centrifuged at 3,273 *g*, and resuspend in LB (BD) + 50% glycerol (SigmaAldrich). Aliquots were made and frozen at -80 °C for experiments. For all assays, starter cultures were subcultured 1:10 into TH + 50 mM HEPES until mid-log phase, which was taken as an absorbance value at OD₆₀₀ of 0.6.

Strain	Notes	Reference
<i>S. pneumoniae</i>		
TIGR4	serotype 4 strain; carry <i>pspC3</i> gene	Rosconi
Δ TIGR4	serotype 4 strain; <i>pspC3</i> deletion	Rosconi

Δcapsule	serotype 4 strain; deletion of SP0350 gene encoding TIGR4 sugar transferase	Rosconi
CT22F 11295	serotype 22F; carry <i>pspC4.1</i> gene	Rosconi
PG27	serotype 35B; carry <i>pspC5.1</i> gene	Rosconi
Taiwan19F-14	serotype 19F; carry <i>pspC6.9</i> gene	Rosconi
PG10	serotype 9V; carry <i>pspC7.2</i> gene	Rosconi
PG24	serotype 3; carry <i>pspC8.2</i> gene	Rosconi
PG12	serotype 6B; carry <i>pspC9.4</i> gene	Rosconi
<i>E. coli</i>		
<i>For carriage of PspC His-tag vectors</i>		
DH5α + pRSET-C. <i>pspC3.4</i>	DH5α carrying pRSET-C. <i>pspC3.4</i> vector	This study
DH5α + pRSET-C. <i>pspC4.1</i>	DH5α carrying pRSET-C. <i>pspC4.1</i> vector	This study
DH5α + pRSET-C. <i>pspC5.1</i>	DH5α carrying pRSET-C. <i>pspC5.1</i> vector	This study
DH5α + pRSET-C. <i>pspC6.9</i>	DH5α carrying pRSET-C. <i>pspC6.9</i> vector	This study
DH5α + pRSET-C. <i>pspC7.2</i>	DH5α carrying pRSET-C. <i>pspC7.2</i> vector	This study
DH5α + pRSET-C. <i>pspC8.2</i>	DH5α carrying pRSET-C. <i>pspC8.2</i> vector	This study
DH5α + pRSET-C. <i>pspC9.4</i>	DH5α carrying pRSET-C. <i>pspC9.4</i> vector	This study
<i>For expression of PspC His-tag proteins</i>		
Rosetta Gami + pRSET-C. <i>pspC3.4</i>	Rosetta Gami expressing rPspC3.4 His-tag protein	This study
Rosetta Gami + pRSET-C. <i>pspC4.1</i>	Rosetta Gami expressing rPspC4.1 His-tag protein	This study
Rosetta Gami + pRSET-C. <i>pspC5.1</i>	Rosetta Gami expressing rPspC5.1 His-tag protein	This study
Rosetta Gami + pRSET-C. <i>pspC6.9</i>	Rosetta Gami expressing rPspC6.9 His-tag protein	This study
Rosetta Gami + pRSET-C. <i>pspC7.2</i>	Rosetta Gami expressing rPspC7.2 His-tag protein	This study
Rosetta Gami + pRSET-C. <i>pspC8.2</i>	Rosetta Gami expressing rPspC8.2 His-tag protein	This study
Rosetta Gami + pRSET-C. <i>pspC9.4</i>	Rosetta Gami expressing rPspC9.4 His-tag protein	This study

Table 5.1. Bacterial strains used in chapter 5

5.2.2 Construction of PspC His-tag vectors

A series of vectors that expressed rPspC-His were constructed (Table 5.2). Primers (Table 5.3) were designed to produce an amplicon encoding a truncated form of *pspC* to insert into the *E. coli* expression vector pRSET-C. The truncated ORF was designed to be composed of the extracellular domain, but not the signal peptide sequence and proline rich regions, allowing expression of recombinant (r)PspC in the cytoplasm of *E. coli* that could be subsequently purified. Each forward primer contained a *Bam*HI restriction site and each reverse primer contained an *Not*I restriction site. PCR was used to amplify the extracellular domains of *pspC3.4*, *pspC4.1*, *pspC5.1*, *pspC6.9*, *pspC7.2*, *pspC8.2*, *pspC9.4* genes from *S. pneumoniae* strains TIGR4, CT22F 11295, PG27, Taiwan19F-14, PG10, PG24, and PG12, respectively, using primers in (Table 5.3) and Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific). The reaction conditions were 12.5 Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific), 9.5 µl of dH₂O, 1 µl forward primer at 20 mM, 1 µl reverse primer (20mM), and 1 µl of *S. pneumoniae* DNA. Standard protocol reaction mixtures of Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific) were used with thermocycling conditions of 2 min 98°C, 35 cycles of 15 s at 98°C, 30 s at 62°C, and 3 mins at 72°C, followed by a final cycle of 5 mins at 72°C. Genomic DNA, kindly provided by the Rosconi group (Boston College), was used as the template. The PCR products were run on a 1% agarose gel and bands were excised and DNA purified using gel extraction kit following manufacturers protocol (QIAquickR Gel Extraction Kit). Subsequently, the PCR product was digested with *Bam*HI (ThermoFischer) and *Not*I (NEB) digestion for 30 mins at 37 °C, and then ligated using DNA ligase (NEB) into linearized pRSET-C vector following manufacturer's protocols. The ligation reaction mixtures were then transformed into *E. coli* DH5α cells, as previously described in Chapter 2, and transformed cells were selected by growth at 37 °C on LB + 100 µg/mL ampicillin agar plates.

Vector	Description	Reference
<i>For expression of recombinant His-tag PspC proteins in E. coli</i>		
pRSET-C. <i>pspC3</i> .His	pRSET-C carrying rPspC3.4 insert under control of T7 promoter	This study
pRSET-C. <i>pspC4</i> .His	pRSET-C carrying rPspC4 insert under control of T7 promoter	This study

pRSET-C. <i>pspC5</i> .His	pRSET-C carrying rPspC5 insert under control of T7 promoter	This study
pRSET-C. <i>pspC6</i> .His	pRSET-C carrying rPspC6 insert under control of T7 promoter	This study
pRSET-C. <i>pspC7</i> .His	pRSET-C carrying rPspC7 insert under control of T7 promoter	This study
pRSET-C. <i>pspC8</i> .His	pRSET-C carrying rPspC8 insert under control of T7 promoter	This study
pRSET-C. <i>pspC9</i> .His	pRSET-C carrying rPspC9 insert under control of T7 promoter	This study

Table 5.2. Vectors used in chapter 5

Oligonucleotide	Sequence (5'-3')
<i>To amplify PspC extracellular domains</i>	
PspC3.4-F	GATCGGATCCACAGAGAAGGAGGTAAC TAC
PspC3.4-R	GAAGAAGATAAAAGTTAAAGCGGCCGCGATC
PspC4-F	GATCGGATCCGAAGGGGTTAGGAGTAAG
PspC4-R	GGCTTAATTCAGAACACAGCGGCCGCGATC
PspC5-F	GATCGGATCCACGGAGAATGAGGGAATTAC
PspC5-R	CTTAAGAAAGCAGTTGATGCGGCCGCGATC
PspC6-F	GATCGGATCCGAAGAGGTTGGTGGTAGG
PspC6-R	GCGCCGGCTCCAAAACCAGCGGCCGCGATC
PspC7-F	GATCGGATCCACAGAGAACGAGGGAAC TAC
PspC7-R	CAAAAAAGGCTTAAC TACAGGACGCGGCCGCGATC
PspC8-F	GATCGGATCCACAGAGAACGAGGGAAGTACC
PspC8-F	CGTCAAGTTCAGGCTCCTCCGCGGCCGCGATC
PspC9-F	GATCGGATCCACAGAGAAAGAGGGAAGTACC
PspC9-F	CAAAAAAGGCTTAAC TACAGGACGCGGCCGCGATC
<i>For screening pRSET-C for inserts</i>	

T7-F	TAATACGACTCACTATAGGG
T7-R	CTAGTTATTGCTCAGCGGT

Table 5.3. Primers used in chapter 5.

Transformant colonies were screened by colony PCR for the presence of a pRSET-C vectors containing an insert that corresponded to the expected fragment size for the respective *pspC* using specific primers (**Table 5.3**) and DreamTaq Green PCR Master Mix 2× (Thermo Fisher Scientific). The following mixture was made 12.5 µl Mastermix + 9.5 µl dH₂O + 1 µl F primer (at 20 mM) + 1 µl R primer (at 20 mM) + 1 µl of template (an *E. coli* colony resuspended in 20 µl dH₂O and boiled for 5 mins) and PCR was set to 95°C for 5 mins, 35 cycles of 95°C for 15 secs, 60°C for 30 secs, 72°C for 60 secs, followed by 72°C for 10 minutes. Strains containing vectors with inserts of the correct size were subsequently cultured and plasmids were purified using the GeneJet® Plasmid Miniprep Kit following the manufacturers protocol. pRSET-C-PspC3, -PspC4, -PspC5, -PspC6, -PspC7, -PspC8, and -PspC9 vectors were sent for sequencing by Eurofins to confirm the sequence of the vector insert.

5.2.3 Expression of recombinant bacterial proteins

Expression and purification of rPspC His-tag proteins

Expression and purification of all rPspC-His proteins was performed as previously described (Chapter 3). To express high volumes of protein, 300 mL of *E. coli* Rosetta Gami (RG) strains carrying pRSET-C vectors (**Table 5.2**) were cultured in LB broth until they reached an absorbance value at OD₆₀₀ of between 0.5-0.7, and then supplemented with 1mM isopropyl β-d-1-thiogalactopyranoside (IPTG, ThermoFisher). After culture for a further 4 hours at 37°C, *E. coli* were harvested by centrifugation of 50 mL of culture in Falcon tubes at 3,273 *g*, and pellets were stored overnight at -80°C. *E. coli* pellets were resuspended in 10 mL native binding buffer (20 mM Na₂HPO₄, 0.5 M NaCl, pH7.8) and two resuspensions were pooled. To lyse the *E. coli*, resuspensions were supplemented with 200 µg/mL lysozyme (SigmaAldrich) and 1mM phenylmethylsulphonyl fluoride (PMSF, ThermoFisher) and incubated on ice for 25 mins. The samples were then sonicated for 10 s at amplitude 50 (ThermoFisher), frozen at -80°C and thawed in a 37°C water bath. 3 cycles of sonication-freeze-thaw were completed. The final *E. coli* lysates were centrifuged at 3,273 *g* to remove cell debris, and the supernatant was filtered using 0.4 µm filters (StarLabs). Proteins were purified by affinity chromatography (ÄKTA pure; GE Healthcare Life Sciences) using 5mL His-Trap Nickel columns (Cytiva, Life Sciences), eluted with gradient imidazole (10-500mM), and dialysed against 1X phosphate buffer saline (PBS, ThermoFisher) at 4°C using 12 kDa

dialysing tubes. Protein samples were concentrated by centrifugation at 3,273 *g* with centrifugal filter units (Merck). The purity of rPspC3.4-His, rPpsC4-His, rPspC5-His, rPspC6-His, rPspC7-His, rPspC8-His, and rPspC9-His was assessed by SDS-PAGE analysis, as previously described (Chapter 2).

5.2.4 Biochemical assays

Binding of LILRB3-Fc to rPspC-His coated Dynabeads

The interaction of LILRB3 with Dynabeads coated with rPspC proteins was measured by flow cytometry. To produce Dynabeads coated with His-tag proteins, 50 μ L of 100 μ g/mL of rPspC3.4-His, rPspC4-His, rPspC5-His, rPspC6-His, rPspC7-His, rPspC8-His or rPspC9-His proteins were incubated with 0.4 μ L of 30 mg/mL Dynabeads His-trap (Invitrogen) for 10 mins at room temperature. The Dynabeads were then washed 3 times in wash buffer (PBS + 0.5% BSA (Sigma) + 10mM Imidazole (SigmaAldrich)) to remove unbound PspC His-tag proteins and blocked by incubating Dynabeads in 30 μ L of PBS + 0.5% BSA (SigmaAldrich) + 10 mM Imidazole (ThermoFischer) for 30 mins on ice. Dynabead washes were performed by addition of 100 μ L wash buffer, transference to a magnet for 2 mins, aspiration of wash buffer, and resuspension in 100 μ L wash buffer. To measure their interaction with LILRB3, 20 μ L of 10 μ g/mL rLILRB3-Fc protein was incubated with 5 μ L of coated Dynabead and incubated on ice for 30 mins, washed 3 times, and stained with 1:1,000 goat anti-human-IgGFc antibody (Southern Biotech), washed 3 times and resuspended in 1% PFA (v/v). The fluorescence of Dynabeads was measured by flow cytometry analysis (FACS Calibur).

5.2.5 Bacterial cell culture assays

Measuring LILRB3-Fc interactions with S. pneumoniae by flow cytometry

The binding of rLILRB3 to *S. pneumoniae* strains was measured by flow cytometry. *S. pneumoniae* TIGR4 wildtype and Δ pspC starter cultures were subcultured 1:10 in Tryptic Soya Broth (Sigma) and grown to mid-log phase (absorbance at OD₆₀₀ = 0.6). To measure rLILRB3-Fc binding to the surface of *S. pneumoniae*, 30 μ L of bacterial cultures (resuspended to an absorbance at OD₆₀₀ of 0.4 in PBS) were incubated with either 10 μ g/ml rLILRB3-Fc or PBS for 30 mins on ice, washed (100 μ L of PBS at 2,250 *g* for 5 mins), and stained with 30 μ L 1:1,000 goat anti-human-IgG-FITC (Life Technologies). To prepare samples for flow cytometry, bacteria were washed and fixed in 1% (v/v) PFA and stored at 4°C. Fluorescence of bacteria was measured by flow cytometry analysis (FACS Calibur).

5.2.6 Human cell culture assays

THP-1 maintenance and differentiation

THP-1 cells (**Table 5.4**) were maintained in RPMI 1640 + GlutaMAX™ (ThermoFisher) supplemented with 10% heat inactivated FCS (Life Technologies). To differentiate cells for adhesion assays and microscopy assays, THP-1 were resuspended to 5×10^5 cells/ml, supplemented with 100 ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 100 μ l added to each well of a 96-flat bottom plate and incubated for 3 days at 37°C in 5% CO₂.

Cell Line	Description	Reference
THP-1	Macrophage-like cell line	Hamon

Table 5.4. Human cell lines used in chapter 5

Immunofluorescence THP-1 infection assay

LILRB3 expression in response to *S. pneumoniae* infection of THP-1 cells was measured by fluorescence microscopy. To produce fluorescently tagged bacteria, *S. pneumoniae* TIGR4 wildtype and Δ pspC starter cultures were subcultured 1:10 in TH (BD) + 50mM HEPES (Sigma) and grown to mid-log phase (OD₆₀₀ = 0.6). To stain bacteria, 1 ml was pelleted by centrifuging at 16, 873 *g* for 1 minute, then resuspended in 1 ml of WGA (ThermoFisher) stain and incubated for 1 hour at 37°C in 5% CO₂. Samples were washed twice in PBS and diluted to 1×10^7 CFU/ml to produce inoculum at MOI 20.

For infection, 100 μ l of inoculum was added to each well of a 96-flat bottom plate containing 5×10^5 THP-1 cells and incubated for 1 hour at 37°C in 5% CO₂. Supernatant was removed, and wells washed twice with 100 μ l PBS, to remove unbound bacteria. Samples were then fixed by addition of 100 μ l of PFA (electron microscope sciences) and incubated for 10 minutes at room temperature.

To measure LILRB3 expression, PFA was aspirated, cells washed twice in PBS, and 50 μ l of mouse anti-LILRB3.IgG2A (R&D Biosystems) at 1 μ g/ml or PBS was added to wells and incubated for 1 hour at room temperature. Subsequently, THP-1 cells were washed twice in PBS, and 50 μ l of DAPI (Invitrogen) and 50 μ l anti-IgG2A antibody (Invitrogen) were added to stain nucleus of THP-1 cells and detect anti-LILRB3 binding,

respectively. Samples were washed and imaged using Cytation 5 (BioTek Society) driven by the Gene 5.1.11 Software. at a magnification of X40.

THP-1 adhesion assay LILRB3 blocking assay

To prepare inocula, *S. pneumoniae* TIGR4 wildtype and Δ pspC starter cultures were subcultured 1:10 in TH (BD) + 50mM HEPES (Sigma) and grown to mid-log phase (absorbance at OD₆₀₀ = 0.6). Cultures were diluted to MOI 0.8 (4×10^4 CFU/100µl) in TH (BD) + 50mM HEPES (Sigma) and 400 µl of 10 µg/ml rLILRB3-His or PBS was added, and cultures were incubated for 30 mins at room temperature to ensure LILRB3 binding.

RPMI media was aspirated from PMA-differentiated THP-1 cells, and 200 µl of inoculum was added to each well of a 96-flat bottom plate in triplicate. The plate was spun at 400 x g for 3 minutes to synchronise adhesion, and then incubated for 1 hour at 37°C and 5% CO₂.

To measure bound/internalised *S. pneumoniae*, 200 µl of dH₂O water was added to each well, vigorously pipetted to lyse cells, then pelleted by centrifuged at 1500 x g for 10 minutes, and finally, resuspended in 100 µl PBS. CFU counts of inoculum and bound/internalised *S. pneumoniae* were measured by serial dilutions of samples and plating onto blood agar plates (Pasteur Institut).

5.2.7 Bioinformatical analysis

Identifying homologous protein sequences using protein Basic Local Alignment Search Tool (BLAST)

Firstly, to identify bacterial proteins that share sequence homology to the B75KN-CTD (**Table 5.5**), protein BLAST was utilised (Altschul et al. 1990). B75KN-CTD was the query sequence against the non-redundant protein sequences (nr) database, with an expected threshold of 0.01 and scoring parameters using BLOSUM62. These settings were used for all subsequent BLAST searches. The BLAST results were filtered to remove *S. agalactiae* proteins and β proteins.

Molecule	Protein sequence	Genbank Accession number	Reference

<i>Reference sequences for initial BLAST search</i>			
B75KN-CTD	NSEMDQAKEKAKIAVSKYMSKVL DGVHQLQKKNH SKIV DLFKELEAIKQQTIFDIDNAKTEVEIDNLVHDAFSKMNATV AKFQKGLETN	ABA44669 .1	(Xu et al. 2022)
CbpA-N	GHMLDSE RDKARKEVEEYVKKIVGESYAKSTKKRHTITVAL VNELNNIKNEYLNKIVESTSESQ LQILMMESRSKVD EAVSK FEK	AAF73792. 1	(Achila et al. 2015)
PspC1	INQEKEKAKLAVVTYM SKILDDIQNIICRKKNIVRL LALIKELDELKKQALSEIDNVNTK VEIENTVHKIFADMDAVVT KFKKGL	AAF73789. 1	(Jarva et al. 2004)
Hic	IDLDRKKAKAAVTEHLKLLNDIEKNLKEQHTHTVELIKNL KDIEKTYLHKLDESTQKAQLQKLIAESQSKLDEAFSKFKNGL	AAG16729	(Jarva et al. 2004)
<i>Consensus sequences for each clade generated by phylogenetic tree</i>			
B75KN-CTD	MDQKEAKIAVSKYMSKLDGVQH LQKNSKIVLFKELEAIKQ QTIFDIDNAKTEVEIDNLVDAFSKMATVAKFQKGL	N/A	This study
CbpA-N	GHMLDSE RDKARKEVEEYVKKIVGESYAKSTKKRHTITVAL VNELNNIKNEYLNKIVESTSESQ LQILMMESRSKVD EAVSK FEK	N/A	This study
PspC1	EKAKLAVTYMSKILDDIQKQIVAIKELDEKKQALSEIDNVNT KVEIENTVHKIF	N/A	This study
Hic	AKLLLDIKNLKKEQHTVELIKLDITYLKDESTKAQLQLESQSK LDEAFSKFKNGL	N/A	This study
Pink Clade	LSLSNHMSELLDR TKKDLKSEESKFDELIKNALGIVNKYRSEL SKAQTK EELNRILETAKSELNATVEEFKKGL	N/A	This study
Yellow Clade	YKSRHTVVNLNIYLNISTSLQLMMSVDAVSFEK	N/A	This study

Table 5.5. Protein sequences used in Chapter 5.

Secondly, a multiple query protein BLAST was performed to identify bacterial sequences with homology to B75KN-CTD, CbpA-N, PspC1, and Hic sequences (**Table 5.5**). To identify the 64 unique sequences, Bioedit was used to removed duplicate proteins and partial peptide sequences from the initial multiple query protein BLAST (Hall 1999).

Finally, a multiple query BLAST analysis of consensus sequences (**Table 5.5**) against PspC variants (**Supp. Table 5.1**) was used to identify PspC variants that contained B75KN-CTD, CbpA-N, PspC1, Hic, pink clade, and yellow clade -like domains. The location of the homologous sequence was used to map potential LILRB3 binding domains on a schematic displaying PspC subtypes and variants.

Generating phylogenetic tree based on 64 unique protein analysis

Phylogenetic analysis was performed using maximum likelihood approach and 1,000 bootstrap replications in MEGA (Kumar et al, 2016). The phylogenetic tree with the highest log likelihood was generated by Maximum Likelihood method and JTT matrix-based model (Jones, Taylor, and Thornton 1992). The percentage of phylogenetic genetic trees generated with associated taxa clustered was calculated. Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model were used to generate initial trees and selecting the topology with superior log likelihood value. The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The phylogenetic tree generated was visualised with interactive tree of life version 4 (iTOL, Letunic & Bork, 2019), and percentage of trees containing associated taxa was display on branches. The likelihood (0, 0.25, 0.5, 0.75, and 1) of cluster association was displayed by size of light purple circles located on branches.

Searching for structural homologs of B75KN-CTD

The Dali sever was used to identify B75KN-CTD structural homologs (Holm et al. 2023). The B75KN-CTD (PDB: 7S0R) file (Xu et al. 2022) was used to search the PDB25 database (a representative subset of the Protein Data Bank) for structural homologs, and the top 10 hits were recorded. Structures were visualised using Pymol.

5.3 Results

5.3.1 The CTD of B75KN shares structural and sequence homology to PspC proteins of *S. pneumoniae*

In Chapter 3, the data showed that LILRB3 bound to B75KN-CTD domain of the β protein expressed by *S. agalactiae*. Given the fact that homologs of β protein can be found in other streptococcal pathogens (such as PspC in *S. pneumoniae*) and homologs of β protein domains can be found in other streptococcal proteins (such as the IgI3 fold found in several streptococcal adhesins), the hypothesis proposed was that other bacterial species may express proteins that contain homologs of B75KN-CTD that interact with LILRB3. First, the protein basic local alignment search tool (BLAST) was used to identify bacterial protein sequences that shared homology to the B75KN-CTD protein sequence (Chapter 2, GenBank accession number: ABA44669.1) (**Fig 5.1A**). After excluding *S. agalactiae* protein sequences and β proteins expressed in other bacteria, the top 10 hits were regions in pneumococcal surface protein C (PspC) that is expressed by *S. pneumoniae* (**Fig 5.1A**).

A

B75KN-CTD sequence analysis

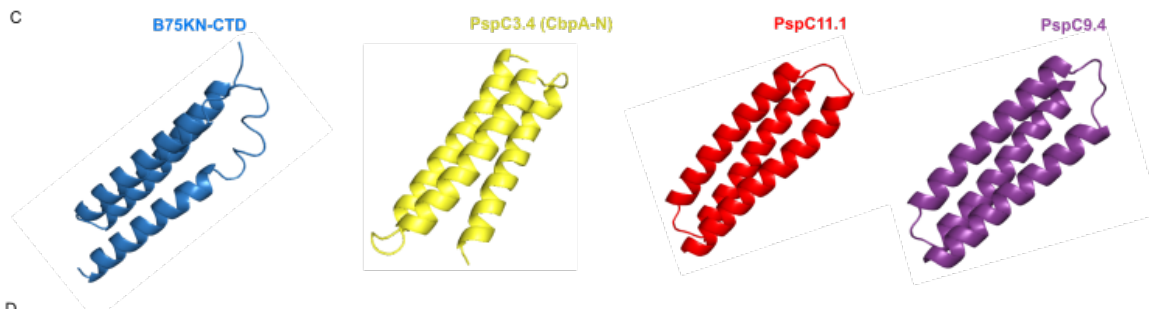
Accession	Species	Protein	Percentage identity to B75KN-CTD (%)
WP_196301003.1	<i>S. pneumoniae</i>	PspC7.4	54.02
WP_193749794.1	<i>S. pneumoniae</i>	PspC9.4	52.87
WP_192854959.1	<i>S. pneumoniae</i>	PspC9.4	54.12
CJA85733.1	<i>S. pneumoniae</i>	PspC9.4	52.87
WP_191867441.1	<i>S. pneumoniae</i>	PspC10.1	52.87
WP_050306825.1	<i>S. pneumoniae</i>	PspC9.4	52.87
VML14135.1	<i>S. pneumoniae</i>	PspC9.2	52.87
COT36117.1	<i>S. pneumoniae</i>	PspC9.4	52.87
CNU37192.1	<i>S. pneumoniae</i>	PspC9.4	52.87
VOF24352.1	<i>S. pneumoniae</i>	PspC9.4	52.87

B

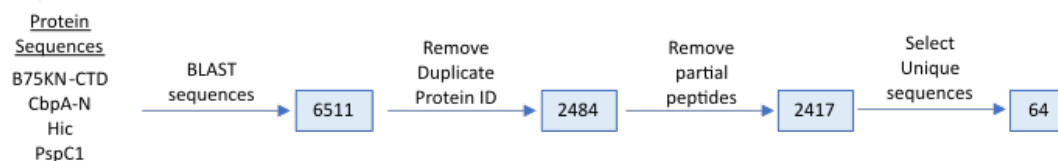
B75KN-CTD structural analysis

Rank	Z score	% ID	Molecule	Class
1	16.2	97	<i>S. agalactiae</i>	c protein beta antigen
2	12.3	27	<i>S. pneumoniae</i>	choline binding protein a (PspC3.4)
3	10.3	9	Rheus cytomegalovirus	rhul123
4	9.2	5	<i>G. sulfurreducens</i>	crispr-associated exonuclease cas4/endonuclease c
5	8.7	5	Homo sapiens	calmodulin-dependent protein kinase
6	8.7	10	Synthetic Human	designed protein 3l6hc2_2
7	8.5	11	cytomegalovirus	immediate early protein 1
8	8.4	7	<i>P. falciparum</i>	presan domain-containing protein
9	7.8	13	<i>M. jannaschii</i>	l-seryl-tRNA(sec) kinase
10	7.7	11	<i>S. cerevisiae</i>	heat shock protein ssa1

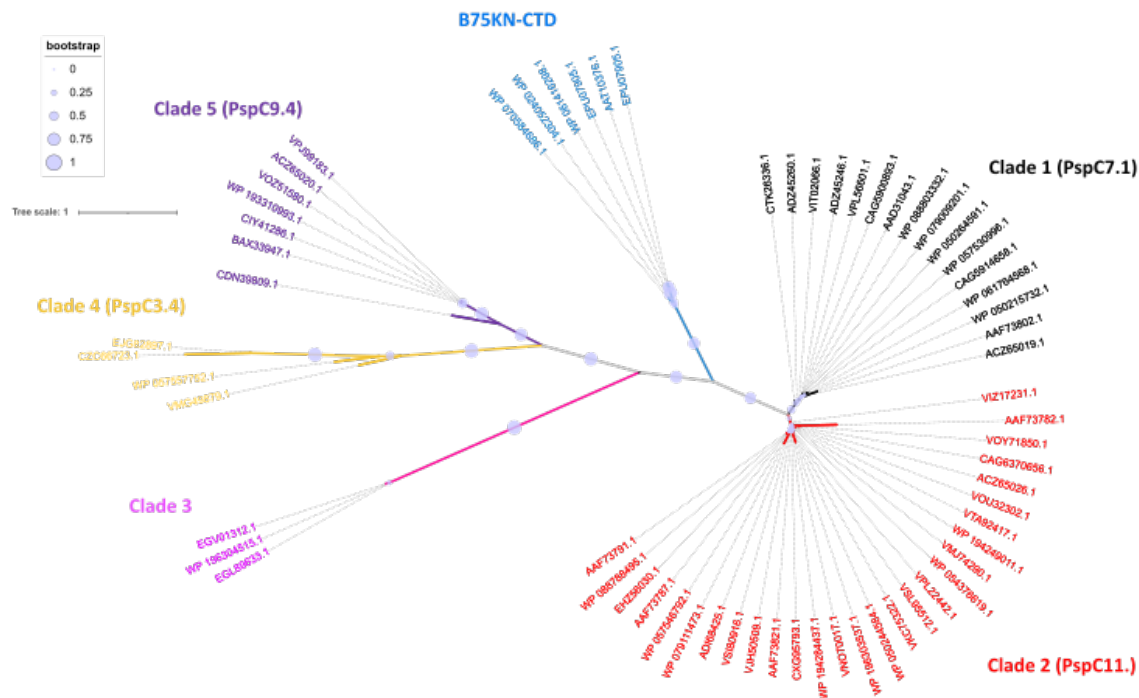
C



D



E



F

Clade	Consensus Sequence
B75KN-CTD	---MDQKEAKIAVSKYMSKLDGVQH--LQKNSKIVLFPKELEAIKQQTIFDIDNAKTEVEIDNLVDAPFSKMA--TVAKFPQK
Clade 1	DINQEKAKLAIAATYMKILDIKKHHLKEKHQIALIKDLRLKQ-ALSEIDNVNTKVL-----
Clade 2 (PspC1)	-----EKAKLAVTYMSKILD-----DIQKQIVAIKELDEKKQ-ALSEIDNVNTKVEIENTVHKIF-----
Clade 3	-LSLSNHMSSEL-LDRTKKDLKSE-----ESKFDELKKNALGIVNK-YRSELSKAQTKEELNRIETAKSELNATVEEFKK
Clade 4 (CbpA-N)	-----YK-----SRHTVVLNLIYLN-IST-SLQLMMSVDA-VSFEK-----
Clade 5 (Hic)	-----AKLLLDI--KNLKK-----EQHTVELIKLDITYLKDESTRAQLQLSESQSKLDEAFSPKNGLL-----

Figure 5.1 B75KN-CTD shares structurally homology to PspC, a highly diverse *S. pneumoniae* cell wall protein. (A) Table displaying the top 10 non-*S. agalactiae* protein sequences that share homology to B75KN-CTD protein sequence using the local Basic Local Alignment Tool (BLAST). (B) Table displaying the top 10 molecules that share structural homology to B75KN-CTD crystal structure. (C) Solved and predicted structures of B75KN-CTD, CbpA-N, PspC1, and Hic. B75KN-CTD (PDB: 7S0R) and CbpA-N (PDB: 3M6U) crystal structures were solved by X-ray crystallography and NMR, respectively. AlphaFold was used to predict PspC1 (Q9K2I5) and Hic (Q9F888) sequences. (D) Schematic of the pipeline used to identify 64 unique proteins from a BLAST of B75KN-CTD, CbpA-N, PspC1, and Hic reference sequences. (E) A phylogenetic tree of the 64 unique protein sequences generated using maximum likelihood approach with a 1,000 bootstrap value using MEGA (molecular evolutionary analysis) and illustrated by iTOL (interactive tree of life). 6 distinct clades for each of the reference sequences were formed B7KN-CTD (blue), clade 1 (black), clade 2 (red), clade 3 (purple), clade 4 (yellow) and clade 5 (magenta). The likelihood (0, 0.25, 0.5, 0.75, and 1) of cluster association is displayed by size of light purple circles located on branches. (F) Consensus sequences for each clade generated using Bioedit software.

PspC – also referred to as CbpA, SpsA, PbcA, or Hic – is a large family of proteins expressed at the surface of *S. pneumoniae* that have functions promoting adhesion and/or immune evasion (Du et al. 2021). Interesting, previous research identified the Hic protein as the β protein homolog in *S. pneumoniae* (Jarva et al. 2002). Historically, PspC/CbpA and Hic proteins were classed as distinct families based on cell attachment (Du et al. 2021). However, a more recent classification based on overall sequence similarity has grouped PspC/CbpA and Hic into one group of *S. pneumoniae* immune evasion proteins that are distinguished into 11 PspC subtypes (PspC1-11). PspC1-6 subtypes comprises the PspC/CbpA subfamily, whilst PspC7-11 subtypes make up the Hic subfamily. Furthermore, variants within each subtype are delineated by the number after the full stop, for example, PspC3.1 and PspC3.2 variants. Thus, the PspC family of proteins will be referred to using the PspC1-11 nomenclature in the remainder of this thesis.

As expected, the PspC variants with the highest sequence homology to B75KN-CTD were from the Hic subfamily – PspC7.4, PspC9.4, PspC10.1, and PspC9.2 (Fig 5.1A). The PspC sequence identity to B75KN-CTD ranged from 54.02-52.87% (Fig 5.1A). To further identify proteins from other bacterial species that contained putative LILRB3-interaction domains, the Dali Server was searched for structural homologs of the B75KN-CTD (Holm et al. 2023) (Fig 5.1B). More specifically, the B75KN-CTD was compared against the exhaustive PDB25 database, a representative subset of the PDB (Protein Data Bank) and the top 10 hits were recorded (Fig 5.1B). As expected, the β protein (C protein β antigen) was the top hit with a Z score of 16.2 and acted as a positive control for the search (Fig 5.1B). The 2nd ranked, with a Z score of 12.3, was PspC3.4 (choline binding protein A, PDB: 2m6u.1) expressed by the TIGR4 strain of *S. pneumoniae*. In conclusion, the bioinformatic analysis indicated that PspC proteins of *S. pneumoniae* contain domains that share sequence and structural homology to the LILRB3 binding domain B75KN-CTD of β protein.

Next, additional PspC proteins that contained a LILRB3 binding domain were identified. Interestingly, the alternative bioinformatical approaches identified PspC proteins of both subtypes, PspC/CbpA (PspC3.4) and Hic (PspC7.4, PspC9.4, PspC10.1, and PspC9.2), that shared sequence or structural homology to B75KN-CTD, respectively. This was expected because, a β protein-like domain is present in PspC/CbpA variant PspC4.2 and Hic variants PspC7.1, PspC10.1, and PspC9.1 (Du et al. 2021). Due to the complexity of the PspC proteins having

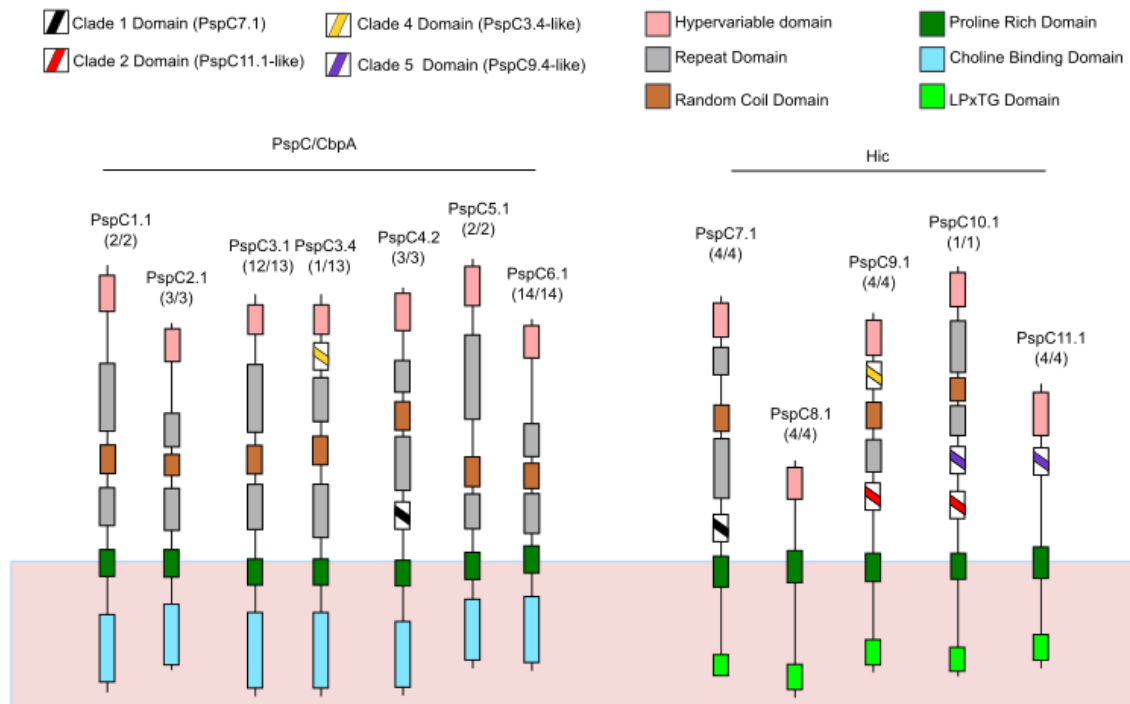
a modular and unique combination of many different domains, determining the PspC variants that contain a potential LILRB3-binding domain was therefore challenging. To overcome this, a BLAST search of B75KN-CTD and 3 PspC reference sequences - PspC3.4 (PspC/CbA; GenBank accession no. AAF73792.1), PspC11.1 (Hic; GenBank accession no. AAG16729), and PspC9.4 (Hic; GenBank accession no. AAF73789.1) - was performed to ensure broad coverage of PspC proteins (Jarva et al. 2002). Interestingly, whilst the B75KN-CTD and the domains from the reference PspC sequences shared low sequence homology, they shared similar structural homology, with each composed of 3 alpha helices connected by two loops (**Fig 5.1C**). Of note, the crystal structure of the N-terminal region of PspC3.4 (CbpA-N) (Achila et al. 2015) was similar to B75KN-CTD. Likewise, the AlphaFold predicted structures of two representative PspC protein sequences – PspC9.4 (AlphaFold accession number: AF-Q9K2I5-F1) and PspC11.1 (AlphaFold accession number: AF-Q9F888) - also displayed this structure (**Fig 5.1C**). The BLAST identified 64 unique protein domain sequences after using inputs of B75KN-CTD, PspC3.4 (CbpA-N), PspC9.4, and PspC11.1, with the expected value threshold set at 0.05 (**Fig 5.1D**).

To assess the evolutionary relatedness of these proteins sequences with B75KN-CTD, an alignment of all 64 unique protein domain sequences was produced and used to construct a phylogenetic tree using MEGA (Tamura, Stecher, and Kumar 2021) (**Fig 5.1E**). Phylogenetic analysis was performed using maximum likelihood approach and a bootstrap value of 1,000, with the likelihood (0, 0.25, 0.5, 0.75, and 1) of cluster association displayed by size of light purple circles located on branches. The protein domains clustered into 6 distinct clades: i) the B75KN-CTD clade, ii) clade 1 iii) clade 2, with domains homologous to reference sequence PspC9.4 (Hic; GenBank accession no. AAF73789.1), iv) clade 3, which does not share sequence homology with any PspC subtype, v) clade 4, with domains homologous to reference sequence PspC3.4 (PspC/CbA; GenBank accession no. AAF73792.1), and vi) clade 5, with domains homologous to reference sequence PspC11.1 (Hic; GenBank accession no. AAG16729). Each clade was strongly supported with bootstrap values of at least 0.75 (**Fig 5.1E**, purple spheres located on each clade branch). Clade 2 exhibited the highest levels of sequence diversity, containing 28 sequences, followed by clade 1, with 16 sequences. Clade 5 and the clade containing B75KN-CTD showed less diversity, containing 7 and 6 sequences, respectively. Finally, clade 3 and clade 4 (containing the domain from CbpA) had low levels of diversity, with 3 and 4 sequences, respectively. However, this diversity could reflect bias in deposition of sequences into databases. Clade 3 appeared the least related to all other clades being clustered the furthest distance from all other clades. The clades 1 and 2 clustered closer together, indicating that they are more closely related to each other than the other 4 major clades within the phylogenetic tree. Interestingly, the B75KN-CTD clade was clustered more closely with clade 1 and clade 2 which contain PspC7.1 and PspC11.1, respectively, than clades 4 and 5 were. This suggests close evolutionary relatedness between the domains in β protein and PspC proteins. In conclusion, PspC proteins are a highly diverse family of related domains that contain at least 4 distinct clades with sequence homology to the B75KN-CTD domain, potentially due to close evolutionary relatedness between β protein and PspC proteins.

5.3.2 LILRB3 binds to a subset of recombinant PspC proteins

Given that the bioinformatic analysis indicated that distinct families of B75KN-CTD-related domains exist in the family of PspC proteins, and because this family of proteins have many domains that are diverse in sequence and organisation, it was important to understand the distribution of potential LILRB3 binding domains across the PspC subtypes. To this end, consensus sequences were generated for each clade derived from the phylogenetic tree to identify potential LILRB3 binding domains (**Fig 5.1F**). An alignment analysis of these B75KN-CTD-related domains was performed against a panel of 55 PspC variants from each of the 11 subtypes (**Supp. Table 5.1**) using protein sequences from genomes kindly provided by the Federico group (Boston College) and those protein sequences previously described in (Du et al. 2021). The location and type of potential B75KN-CTD domain was shown on representative PspC variants (**Fig 5.2A**, Du et al. 2021). There are 11 PspC subtypes based on specific domains: hypervariable, repeat, random coil, and proline rich domains. PspC1 to PspC5 subtypes, make up the PspC/CbpA subfamily, and express a choline binding domain that non-covalently binds choline present in the cell wall. In contrast, PspC6 to PspC11 make up the Hic subfamily, contain an LPxTG domain that is covalently attached via sortase to the cell wall (**Fig 5.2A**) (Du et al. 2021). The mapping of B75KN-CTD-related domains to representative PspC subtypes is shown in **Fig 5.2A** with details on whether the B75KN-CTD-related domain belongs to the B75KN-CTD clade, clade 1, clade 2, clade 3, clade 4 or clade 5. Furthermore, how representative each variant is of the subtype is depicted by the number in brackets (the numerator is the number of variants being represented; the denominator is the total number of variants screened) (**Fig 5.2A**). A Clade 4 domain, which contains sequences most homologous to PspC3.4, was present in PspC3.4 as expected. Interestingly, all other PspC3 subtypes (12/13) represented by PspC3.1 did not contain a clade 4 domain, suggesting that variants within subtypes have different phenotypic properties. A Clade 1 domain, which contains sequences most homologous to PspC7, was present in all PspC4 (3/3) and PspC7 (4/4) subtypes; and a clade 5 domain was present in all PspC11 subtypes (4/4). Interestingly, certain subtypes contained multiple B75KN-CTD related domains. PspC9 subtypes contained a clade 4 and clade 2 domain, whilst the PspC10.1 variant expressed a clade 5 and clade 2 domain. Thus, specific PspC subtypes/variants express different B75KN-related domains. The analysis identified several variants from different subtypes containing different B75KN-related domains (17/57). However, PspC1, PspC2, PspC5, PspC6, and PspC8 contained no variants with sequences homologous to any B75KN-related domains. This may reflect the diversity of the PspC proteins, or the paucity of variant sequences to screen; for example, only two PspC1 variants were screened (**Fig 5.2A**). No PspC variant contained clade 3 or B75KN-CTD domains. This is perhaps unsurprising, because clade 3 is more phylogenetically distinct from the other clades, whilst B75KN-CTD domain clade is composed of β proteins (**Fig 5.1E**).

A



B

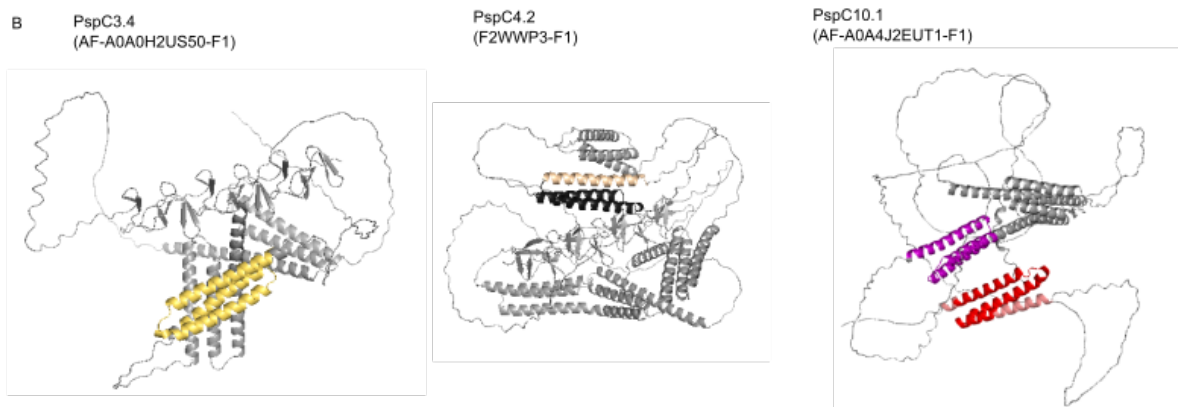


Figure 5.2 PspC subtypes express domains homologous to clade consensus sequences. (A) Schematic of representative PspC subtypes 1-11 with key domains highlighted. Adapted from (Du et al. 2021). Domains that share homology to consensus clade sequences are shown in boxes containing coloured strips: clade 1 (black), clade 2 (red), B75KN-CTD-like (blue), clade 4 (yellow) and clade 5 (purple). The number of variants being represented over the total variants screened from that subtype is shown in brackets after PspC variant e.g. PspC1.1 represents 2 variants of the 2 variants screened **(B)** AlphaFold structures of three PspC variants – PspC3.4, PspC4.2 and PspC10.1 with potential LILRB3 binding domains highlighted in yellow (clade 2), black (clade 1), purple (clade 5), or red (clade 2).

To determine whether the different B75KN-related domains mapped to the common 3 alpha helical structure exhibited by the reference strains (**Fig 5.1C**) the AlphaFold database was searched for predictive PspC structures

and the domains highlighted that had homology to B75KN-related domains (**Fig 5.2B**). The clade 4 domain (yellow) mapped to a 3 alpha helical structure in PspC3.4 (**Fig 5.2B**). In PspC4.2, clade 1 (black) mapped partially to a 3 alpha helical structure (**Fig 5.2B**). Finally, clade 2 (red) and clade 5 (purple) domains mapped to 3 alpha helical structures in PspC10.1 (**Fig 5.2B**). Thus, whilst the B75KN-related domains differ in sequence, forming distinct clades, they may share similar secondary structures. In conclusion, several PspC variants/subtypes contained B75KN-CTD-related domains that mapped to a 3 alpha helical structure which may potentially interact with LILRB3. Indeed, certain PspC variants expressed more than one B75KN-CTD-related domain. However, the distribution of B75KN-CTD-related domains among PspC variants did not appear to be dependent on the PspC subfamily (CbpA/PspC or Hic subfamily) or subtype (PspC1-11).

Since our bioinformatical analysis suggested a wide range of PspC proteins may interact with LILRB3, the aim was to produce recombinant proteins for each PspC subtype (1-11), to ensure broad coverage, and subsequently measure each for LILRB3 binding. To do this, DNA encoding the PspC extracellular domains was amplified from *S. pneumoniae* genomic DNA kindly provided by Federico Rosconi (Boston College), and *E. coli* vector and protein expression systems were used to produce recombinant (r)PspC-His proteins. Using PspC3.4 as an example, primers were designed to amplify the region between the signal peptide and proline rich domain of TIGR4 DNA. PCR products were run on an agarose gel that showed amplicons at the expected size of ~1.4 kB (**Fig 5.3A**).

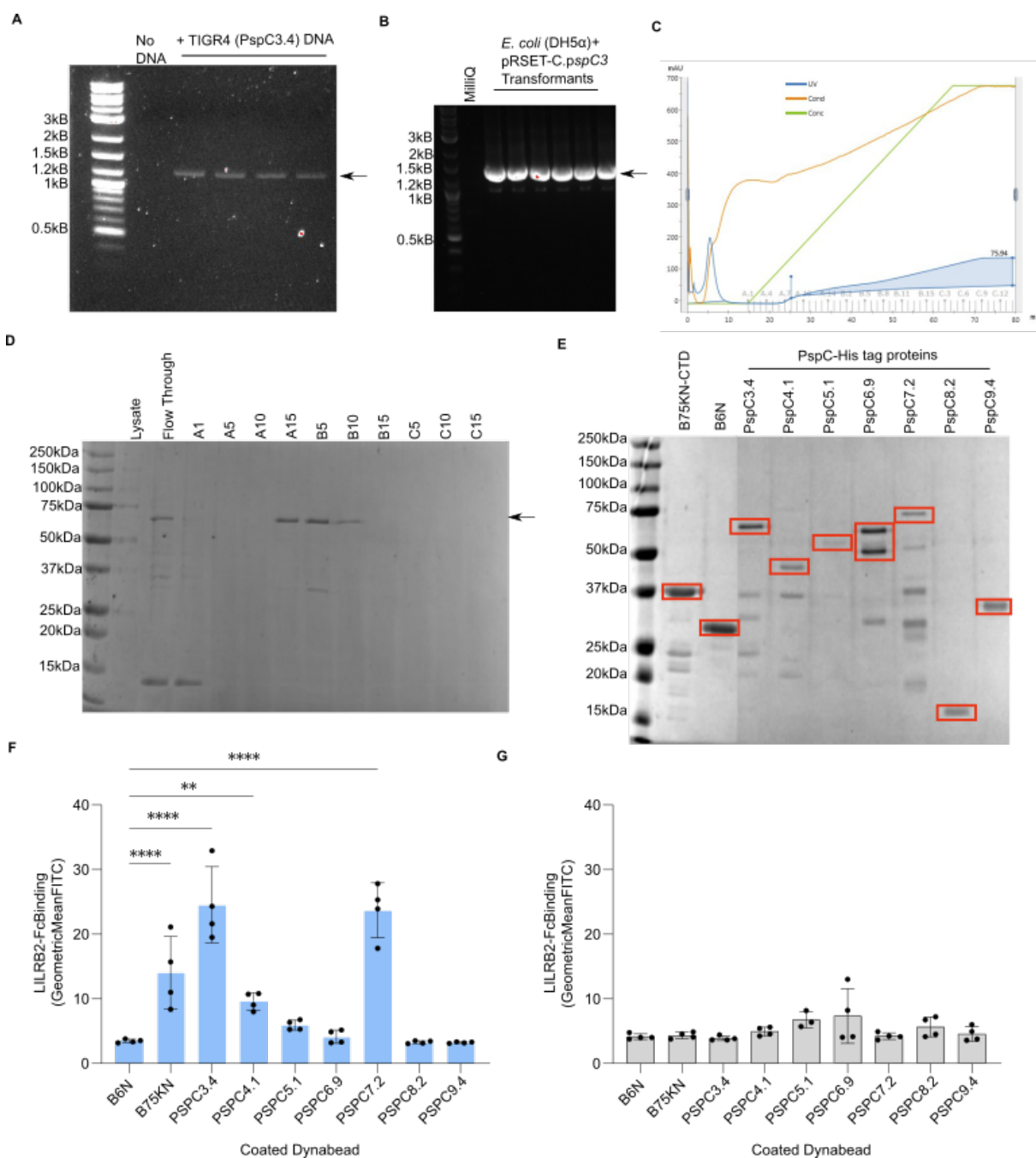


Figure 5.3 LILRB3 binds specific PspC subtypes. (A-D) Construction of recombinant (r)PspC3-His as an exemplar for constructing rPspC-His proteins. **(A)** *pspC3* extracellular domain amplicons analysed by agarose gel. Amplicons were produced by PCR of TIGR4 strain template DNA, using primers flanking the extracellular domain of *pspC3*. **(B)** Insertion of *pspC3* amplicon into pRSET-C. Transformed colonies were assessed by colony PCR for carriage of pRSET-C vectors with *pspC3* inserts. **(C-D)** Purification of rPspC3-His by ATKA™ protein purification system. *E. coli* lysate was passed through His-trap columns, which were eluted using an imidazole gradient (10-500 mM). The absorbance of elution fractions **(C)** was used to determine the elution samples that were tested for protein expression by SDS-PAGE **(D)**. **(E)** SDS-PAGE of rPspC-His proteins (rPspC3-His, rPspC4-His, rPspC5-His, rPspC6-His, rPspC7-His, rPspC8-His, and rPspC9-His) to assess for purity. B75KN-CTD and B6N were used as positive controls. Red boxes outline bands that are at the expected kDa weight for each protein based on its sequence, as calculated in EXPASY. **(F-G)** LILRB3-Fc binding to rPspC-His was assessed by flow cytometry. rPspC-His coated Dynabeads were incubated with rLILRB3-Fc **(F)**, or rLILRB2-Fc **(G)**, then washed, and stained for rLILR-Fc binding using goat anti-human-IgG-FITC (1:1,000). Experiments were performed with 4 biological replicates. One-way ANOVA was performed to assess statistical significance (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.001$).

PspC3 amplicon bands were excised, purified and digested with *Bam*HI and *Not*I. The digested fragments were then ligated into a pRSET-C vector that had been digested with *Bam*HI and *Not*I, and the constructed vector was used to transform *E. coli* DH5 α . To identify transformant colonies containing pRSET-C vector with a correct *pspC3* sequence, colony PCR was performed using primers designed to amplify across the multiple cloning site (MCS) of pRSET-C (**Fig 5.2B**). All colonies showed amplification of the insert with bands at ~1.4 kDa, thereby, confirming presence of the *pspC3* insert in pRSET-C (**Fig 5.2B**). A pRSET-C.*pspC3* vector clone was grown overnight and miniprepmed to produce a high concentration of purified plasmid that was sent for sequencing to confirm the presence of an open reading frame encoding rPspC3.4-His. Having confirmed correct sequence, pRSET-C.*pspC3.4*, was transformed into *E. coli* RG. rPspC3-His was overexpressed in RG *E. coli* carrying pRSET-C.*PspC3* and was purified from the *E. coli* lysate by affinity chromatography (**Fig 5.3C**). There was no clear peak in absorbance during the elution process, to guide an estimation of the elution fractions containing rPspC3-His. Therefore, to identify elution fractions containing rPspC3 every 5th elution fraction was tested for the presence of proteins by SDS-PAGE analysis. This showed that fractions A15, B5, and B10 contained bands at the expected molecular weight of PspC3-His at ~60 kDa (**Fig 5.3D**). Therefore, elution fractions A15 to B10 were pooled, dialysed into PBS overnight at 4°C, and concentrated using 10kDa filter columns.

In addition, rPspC4-His, rPspC5-His, rPspC6-His, rPspC7-His, rPspC8-His, and rPspC9-His proteins were expressed and purified using the method described above. rPspC1-His, or rPspC11-His were not expressed due to the lack of the *S. pneumoniae* strains that expressed these PspC subtypes. Furthermore, rPspC2-His or rPspC10-His were not expressed due to primers designed not amplifying the correct insert after vectors were sent for sequencing. SDS-PAGE was used to assess purity of all rPspC-His proteins, with red boxes indicating the band containing full length PspC (**Fig 5.3E**). More specifically, the predicted molecular weights were: rPspC3 (~48 kDa), rPspC4 (~62 kDa), rPspC5 (~71 kDa), rPspC6 (~46 kDa), rPspC7 (~58 kDa), rPspC8 (~15 kDa), and rPspC9 (~30 kDa). Whilst several of the rPspC bands migrated to lower or higher than predicted, previous literature suggests predicting migration through SDS using molecular weight is unreliable, possibly due to post-translational modification such as glycosylation (Jedrzejas 2001). In addition, there were multiple bands at lower molecular weights produced

for most of the purified rPspCs. This is likely because of protein degradation, potentially due to repeated thawing and/or degradation during the purification process, which was also true in the positive controls B75KN-CTD and B6N (**Fig 5.3E**). Alternatively, this could indicate that there were contaminant proteins in the rPspC preparations, though this is less likely as it could be anticipated the same contaminants would be in all purification given that the expression conditions and purification process was standardised. Furthermore, there was variation in the thickness of rPspC bands suggesting variable concentrations between subtypes. For example, rPspC5 appear to have a lower concentration than other rPspC subtypes.

Next a screen of the rPspC-His proteins for their capacity to LILRB3 was performed. To test this, rPspC-His coated Dynabeads were incubated with rLILRB3-Fc or rLILRB2-Fc, and the binding of rLILR proteins was measured by flow cytometry (**Fig 5.3F-G**). B75KN-CTD and B6N coated Dynabeads were used as positive and negative controls for LILRB3-Fc binding, respectively. rLILRB3-Fc bound to rPspC3-, rPspC7-, and rPspC4-coated Dynabeads significantly more than B6N Dynabeads (**Fig 5.3F**). In contrast, rLILRB3-Fc did not bind rPspC5, rPspC6, rPspC8, and rPspC9 Dynabeads. Of note, rPspC3- and rPspC7-coated Dynabeads had higher levels of LILRB3-Fc binding compared with PspC4-coated Dynabeads. Interestingly, rLILRB3-Fc bound rPspC3 and rPspC7 Dynabeads more than the positive control, rB75KN-CTD. LILRB2-Fc did not bind to any of the rPspC proteins, showing that LILRB3-Fc binding was specific (**Fig 5.3G**). Therefore, the data suggested that LILRB3 directly binds to PspC3, PspC7, and PspC4.

5.3.3 *S. pneumoniae* TIGR4 does not interact with LILRB3 to enhance adhesion

As the data suggested that rPspC3, rPspC4, and rPspC7 directly interact with LILRB3, the hypothesis proposed was that *S. pneumoniae* expressing these PspC subtypes interact with LILRB3. This hypothesis was addressed by using the TIGR4 strain because it contains the gene encoding PspC3.4 and is one of the best-characterised virulent *S. pneumoniae* strains. PspC3.4 is a key virulence factor that promotes immune evasion and colonisation as it binds to a plethora of host ligands including FH, C4BP, C3b/iC3b, and IgA/plgR (Yu et al. 2018; Zhang et al. 2000; Achila et al. 2015) (**Fig 5.4A**). PspC3.4 is thought to promote immune evasion of TIGR4 in blood through inhibiting complement activation by binding FH and C4BP (Marshall et al. 2021; Dieudonné-Vatran et al. 2009) and is thought to promote colonisation capacity of TIGR4 through binding IgA/plgR (Yu et al. 2018). Thus, the hypothesis was that *S. pneumoniae* TIGR4 may interact with LILRB3 to promote adhesion and/or immune evasion.

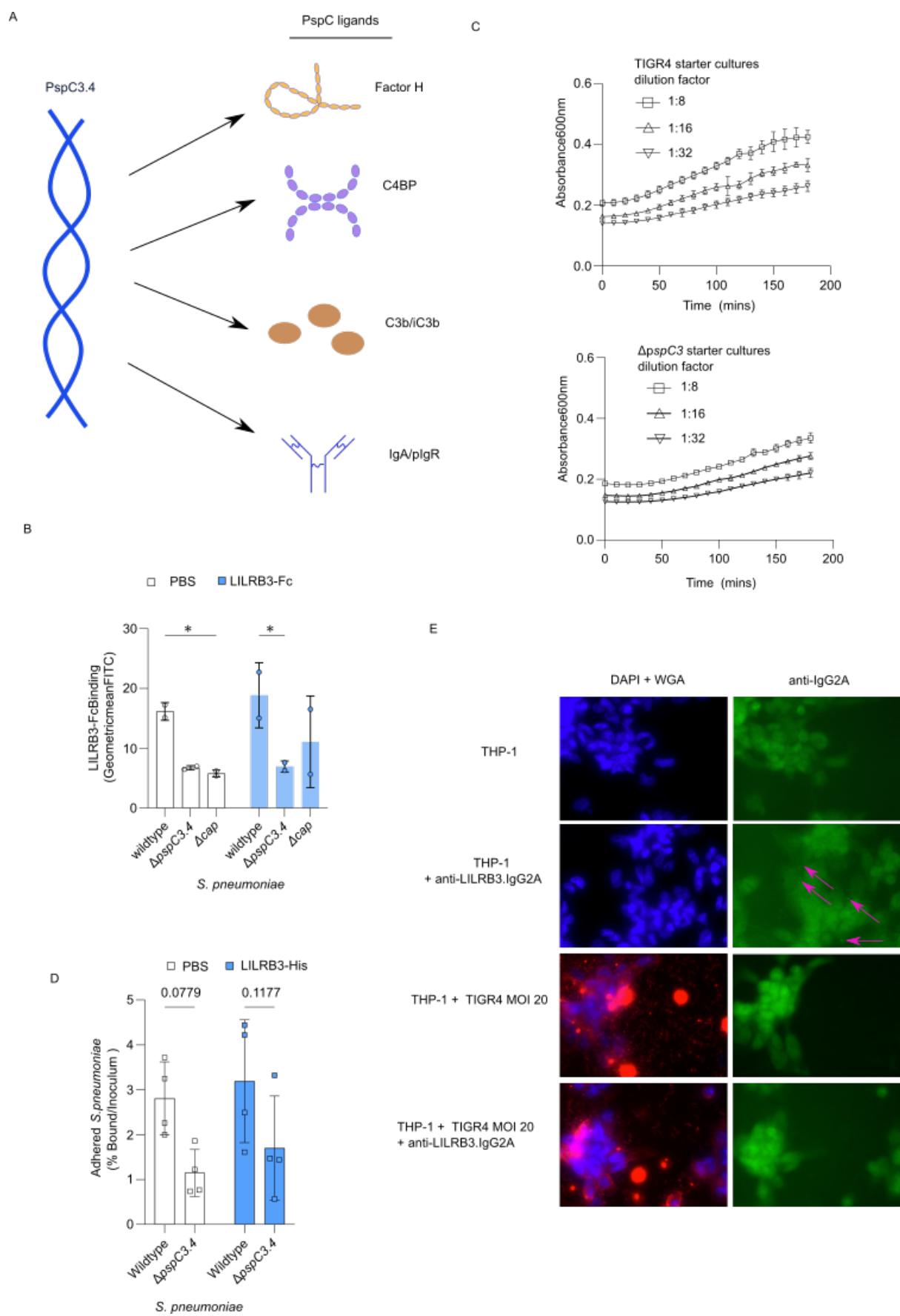


Figure 5.4 PspC3 mediates *S. pneumoniae* adherence to THP-1 cells independent of LILRB3. (A) Schematic showing host ligands known to interact with PspC proteins. **(B)** Binding of recombinant LILRB3-Fc to the surface of *S. pneumoniae* strains was measured by flow cytometry. Mid-log phase *S. pyogenes* wildtype, Δ pspC3 and Δ cap TIGR4 strains (OD₆₀₀ = 0.6) were incubated with LILRB3-Fc (10 µg/ml) or PBS for 30mins, washed in PBS, stained with goat anti-human-Fc-FITC mAb (1:1,000) for 30mins. Samples were then washed in PBS and fixed in 1% PFA. Data points represents $n = 2$ independent experiments. **(C)** Growth curves of wildtype and Δ pspC3 TIGR4 strains. Starter cultures at OD₆₀₀= 3 were serially diluted 1:8, 1:16, and 1:32 into TH + HEPES to final volume of 100 µl and absorbance was measured every 10 mins for 180 mins by Cytation 5. **(D)** LILRB3-His blocking of PspC3-mediated TIGR4 adhesion to THP-1 cells. 5×10^4 THP-1 cells were differentiated using 100ng PMA (1:10,000) for 3 days, then THP-1 cells were infected with at MOI 20 of TIGR4 or Δ pspC3 strains preincubated with LILRB3-His (10µg/ml) or PBS for 1 hour. THP-1 cells were washed in PBS to remove unbound bacteria and lysed in dH₂O water. CFU counts were measured of serially diluted THP-1 lysates and bacterial inocula samples and % adherence was calculated, $n = 4$ biological replicates. **(E)** Representative images detecting LILRB3 surface expression on PMA-differentiated THP-1 cells after 1 hour challenge with TIGR4 (MOI 20), or uninfected (UI) by fluorescence microscopy. Mid-log phase TIGR4 were stained with WGA-red (100 ng/ml) for 1 hour at 37 C prior to 1 hour challenge of THP-1 cells. Post challenge, THP-1 cells were washed twice with PBS, and fixed in 4% PFA. Cell were stained with mouse anti-LILRB3-IgG2A, or incubated in PBS for 1 hour at 37 C, and washed twice in PBS. Finally, THP-1 cells were stained with DAPI and anti-IgG2A (1:1,000) for 1 hour at 37 C, and imaged at X40 magnification using Cytation 5. Two-way ANOVA was used to determine statistical significance **(B,D)** (* $p < 0.05$) .

To first assess TIGR4 and LILRB3 interactions, LILRB3-Fc binding to wildtype TIGR4 and isogenic PspC knockout strain (Δ pspC3) and acapsular strain (Δ cap) was tested (**Fig 5.4B**). The isogenic TIGR4 strains were kindly provided by the Rosconi lab and deletion of PspC3.4 and capsule were verified by sequencing. Whilst LILRB3-Fc bound significantly more to TIGR4 wildtype than Δ pspC3, this difference was also observed for the PBS control (**Fig 5.4B**). The differences in background signal for the wildtype and Δ pspC3 strains could be due to differences in non-specific binding of the secondary antibody. LILRB3-Fc binding to the Δ cap strain was reduced compared with wildtype (**Fig 5.4B**), suggesting that the secondary antibody may be binding to the capsule. Nonetheless, the data suggested that LILRB3-Fc does not directly bind to the surface of *S. pneumoniae* TIGR4 strain. It is possible that surface expression of PspC3 in this strain, or under these experimental conditions, is low. In support of this, PspC3 levels are known to be modulated under different experimental conditions, as microarray data has shown that TIGR4 pspC3 is upregulated upon interaction with host epithelial cells (Orihuela et al. 2004). Thus, LILRB3-PspC3 interaction in TIGR4 may be more easily detected using human cell line infection models.

To assess whether TIGR4 forms an interaction with LILRB3 during human cell infection, a THP-1 infection model using TIGR4 *S. pneumoniae* strains established in the Melanie Hamon lab was utilised (Pasteur Institut, Paris). THP-1 is a monocytic cell line that has been shown to express surface LILRB3 (Yu et al. 2018). First, the growth rates of isogenic TIGR4 wildtype and Δ pspC3 starter cultures were measured, to ensure that they exhibited similar mid-log phases of growth (**Fig 5.4C**). This was important because at an absorbance at OD₆₀₀ of 0.6, TIGR4 wildtype had ~3.5 times lower CFU count than Δ pspC3 (TIGR4 wildtype = 1×10^8 CFU/ml, Δ pspC3 = 3.375×10^8 CFU/ml). To measure growth, starter cultures at OD₆₀₀= 3 of TIGR4 wildtype and Δ pspC3 were diluted 1:8, 1:16, and 1:32 to a final volume of 200 µl growth media and absorbance at OD₆₀₀ was measured every 10 minutes for 180 minutes. Wildtype and Δ pspC3 strains were in mid-log phase over 180 minutes, with neither growth curve

plateauing (**Fig 5.4C**). There was only a slight lag phase observed for each strain, as expected when using starter cultures. Notably, Δ *pspC* samples had consistently lower absorbance values at OD₆₀₀ compared to the TIGR4 wildtype strain at equivalent dilutions suggesting that PspC3 expression may be responsible for differences in absorbance. Thus, TIGR4 wildtype and Δ *pspC3* exhibited similar growth rates and were suitable for THP-1 infection assays.

Having established growth and culture conditions of the TIGR4 strains for THP-1 infection-based assays, the next assays determined whether rLILRB3-His could block the *S. pneumoniae* interaction with THP-1 cells (**Fig 5.4D**). It would be expected that pre-incubation of TIGR4 with rLILRB3 would reduce LILRB3-dependent adhesion to THP-1 cells. To perform these assays, THP-1 cells were differentiated for 72 hours in PMA to produce adherent macrophages, then infected with an MOI 0.8 of TIGR4 wildtype or Δ *pspC3* for 1 hour at 37°C + 5% CO₂. THP-1 cells were subsequently washed to remove unbound bacteria, and the THP-1 cells were lysed, diluted and plated onto TH + HEPES agar plates to measure adherent bacteria. To assess the role of rLILRB3-His blocking, TIGR4 wildtype and Δ *pspC3* inocula were resuspended in PBS or rLILRB3-His for 30 mins prior to infection. For inocula resuspended in PBS, the wildtype TIGR4 adhered better than the Δ *pspC3* strain to THP-1 cells; however, the difference was not statistically significant (**Fig 5.4D**). This data suggested that PspC3 acts as an adhesin (Zhang et al. 2000) which is consistent with previous findings (Iovino et al. 2017). However, incubation of rLILRB3-His did not block the adhesion of wildtype TIGR4 to THP-1 cells. This suggested that PspC3-LILRB3 interactions do not promote *S. pneumoniae* adhesion to macrophages under the experimental conditions assessed.

To further investigate PspC3-LILRB3 interactions, a pilot experiment was performed to assess whether *S. pneumoniae* co-localises with LILRB3 expressed on THP-1 cell surfaces using fluorescence microscopy (**Fig 5.4E**). In these assays, PMA-differentiated THP-1 cells were infected with wheat germ agglutinin (WGA) fluorescently stained wildtype TIGR4 strain at an MOI of 20 for 1 hour at 37°C + 5% CO₂. WGA binds to sialic acid residues present on bacterial surfaces enabling localisation of bacteria. Subsequently, THP-1 cells were washed to remove unbound bacteria and fixed. Finally, the cell cultures were stained with DAPI to localise the THP-1 nucleus and with a monoclonal antibody against LILRB3 to localise LILRB3. THP-1 cells not stained with anti-LILRB3 acted as negative controls (row 1, row 3, **Fig 5.4E**). The fluorescence microscopy images did not detect any binding of the anti-LILRB3 mAb to THP-1 cells infected with TIGR4 (bottom right panel, **Fig 5.4E**), when compared to the negative control THP-1 cells solely stained with secondary antibody (middle bottom right panel, **Fig 5.4E**). Interestingly, uninfected differentiated THP-1 cells stained for LILRB3 expression exhibited several puncta that are highlighted with arrows (middle top right panel, **Fig 5.4E**). These puncta suggested that LILRB3 is expressed on the surface of differentiated THP-1 cells under normal conditions. However, no puncta were detected in THP-1 cells infected with TIGR4, suggesting that surface expression of LILRB3 is downregulated by THP-1 cells during *S. pneumoniae* infection.

5.4 Discussion

The key discovery in this exploratory chapter was that a subset of PspC proteins expressed by *S. pneumoniae* may interact with LILRB3. Having previously discovered that β protein expressed by *S. agalactiae* interacts with LILRB3 in Chapter 3, this knowledge was utilised to identify a subset of PspC proteins expressed by *S. pneumoniae* that also potentially bind LILRB3. These data suggest that LILRB3 binding is conserved by multiple streptococcal species and supports the proposition that LILRB3 is an important target for bacterial pathogens. Identifying LILRB3 ligands is essential for determining whether bacteria target LILRB3 for immune evasion, or if the interaction may enhance host responses to bacterial infections. Elucidating the role of bacterial interactions with LILRB3 will deepen understanding of streptococcal pathogenesis, and potentially identify common mechanisms utilised by multiple streptococcal species. More specifically, investigating the LILRB3-PspC interaction may reveal additional functionality for PspC proteins, a highly diverse family of cell surface choline binding proteins that are important virulence factors associated with invasive disease (van der Maten et al. 2018). Whilst PspC proteins are known to function as adhesins, they are also proposed to be immune evasion molecules due to their ability to sequester FH and C4BP to inhibit complement activation (Dieudonné-Vatran et al. 2009; Dave et al. 2001; Haleem et al. 2018). However, the functional consequences of PspC expression for bacterial survival are yet to be fully elucidated. Thus, discovery of a potential PspC-LILRB3 interaction provides a novel avenue for fully characterising the immune evasion function proposed for PspC proteins.

In this chapter, a bioinformatical approach was taken to identify novel LILRB3 ligands using β protein structural and sequence data previous described in Chapter 3. Several genomic studies have revealed streptococcal pathogens, particularly *S. pneumoniae*, *S. agalactiae* and *S. pyogenes*, are closely related sharing hundreds of putative proteins (Yu et al. 2018; Takeda and Nakamura 2017). Indeed, *S. pneumoniae* PspC proteins of the Hic subfamily were proposed homologs of β protein (Jarva et al. 2002) raising the prospect that these proteins could interact with LILRB3. In support of this, the bioinformatic analysis data identified that sequence or structure homologs of the B75KN-CTD domain are present in the PspC family of proteins expressed by *S. pneumoniae*. Of note, the presence of the B75KN-CTD-related domain was independent of the subfamily – PspC/CbpA and Hic – and PspC1-11 subtype. Thus, several rPspC proteins were constructed that were representative of a range of subtypes as determined by (Du et al. 2021). This LILRB3 binding screen identified 3 hits rPspC3.4, rPspC7.2 and rPspC4.1. The other PspC – PspC5.1, PspC6.9, PspC8.2, PspC9.4 - showed no interaction with LILRB3 in this experimental set-up. However, low LILRB3 binding to PspC5.1 may be due to low levels of protein concentration. In future work, it would be useful to reduce amounts of freeze/thawing steps and optimise protein pH conditions to reduce levels of degradation. Collectively, these data suggest that the presence of a putative LILRB3 binding domain in PspC proteins is variant specific. In addition, the results reveal that expression of a LILRB3 binding domain is independent of choline binding domain (CbpA/PspC) or LPxTG domain (Hic) anchoring. Furthermore, LILRB3 binding is not dependent on expression of B75KN-CTD, clades 1, clade 2, clade 3, clade 4, or clade 5-like domains; for example, PspC3.4 and PspC9.1 both expressed a clade 4 domain, but only PspC3.4 bound LILRB3.

This indicates potential sequence and/or structural differences in clade-like domains, suggesting a limitation of this approach for identifying LILRB3 binding domains. Nevertheless, the expression of a clade 4 domain in PspC3.4 may indicate the potential location of the LILRB3 binding domain in the N-terminal region. Interestingly, the clade 4 domain was not identified in the other 12 PspC3 variants analysed (PspC3.1-PspC.13) suggesting that expression of potential LILRB3 binding domains is variant specific and independent of PspC subtype. The other two hits were PspC4.1 and PspC7.1, which both contain a clade 1 like domain. Thus, PspC4.1 and PspC7.1 may express alternative LILRB3 binding domains to PspC3.4, and similar to β protein, suggests multiple LILRB3 binding domains in PspC proteins which be present. Unfortunately, some PspC proteins were not expressed and purified, namely PspC1.1, PspC2.1, PspC10.1 and PspC11.1. Notably, PspC10.1 and PspC11.1 contain clade 5 -like domains that would be worth testing for LILRB3 binding. Therefore, it is of interest in future studies to assess the interaction of these PspC subtypes with LILRB3.

Having identified 3 PspC subtypes that bound LILRB3, the LILRB3 interaction with PspC3.4 was explored next. This protein was taken forward to further investigate LILRB3 interaction because it is expressed by the well characterised TIGR4 strain. The TIGR4 strain is highly virulent clinical isolate that causes extremely high mortality rates in mouse models, even compared with other virulent strains (Yau et al. 2017). A plethora of key virulence factors contribute to TIGR4 virulence with the best characterised being polysaccharide capsule, pneumococcal surface protein A (PspA) and PspC (Ricci et al. 2013). Despite the biochemical evidence indicating that PspC3.4 and LILRB3 interact, no LILRB3-Fc binding to wildtype TIGR4 was detected by flow cytometry. One explanation is that the TIGR4 strain does not express sufficient levels of PspC to detect LILRB3-Fc binding. Therefore, to fully interpret the data, it would be important to generate anti-PspC serum or use a mAb against PspC3.4 to measure surface expression of PspC3.4 by flow cytometry. Alternatively, TIGR4 may express surface structures that block LILRB3-Fc interaction with surface expressed PspC3.4. In future assays, assessing LILRB3-Fc interaction with chemically treated TIGR4 to remove glycosylation and/or protein surface structures may reveal a LILRB3-TIGR4 interaction.

These investigations exploring *S. pneumoniae* adhesion to THP-1 cells in an infection model also did not reveal a role for LILRB3. PspC-mediated adhesion of TIGR4 to THP-1 cells was not blocked by pre-incubation of the strains with LILRB3-His. These results were therefore consistent with the data showing that rLILRB3-Fc did not bind to the surface of *S. pneumoniae*. Indeed, the inability of rLILRB3-His to inhibit *S. pneumoniae* adhesion to THP-1 cells may be a result of rLILRB3-His not engaging PspC on the bacterial surface. The data generated here do support the role of PspC3.4 as an adhesin, as PspC3.4 expression enhanced *S. pneumoniae* TIGR4 adhesion to THP-1 macrophages. The proposed mechanism for PspC-mediated adhesion to THP-1 cells is through upregulation of expression of intracellular adhesion molecule 1 (ICAM-1); however, this has not been shown experimentally (Thornton and McDaniel 2005). These results indicated that this was not through binding LILRB3. One explanation for why PspC3.4-LILRB3 interactions did not impact *S. pneumoniae* TIGR4 adhesion to THP-1 cells may be that the differentiated THP-1 cells expressed low levels of surface LILRB3. Though undifferentiated THP-1 cells express LILRB3 (Yu et al. 2018), the culture of THP-1 cells at a range of PMA concentrations and incubation times have been shown to induce different THP-1 receptor expression profiles and levels of bacterial

interactions (Starr et al. 2018). As it is currently unclear if 3-day PMA-differentiation alters surface expression of LILRB3, future assays are required to measure LILRB3 expression over time and at a range of PMA concentrations during differentiation. Alternatively, to overcome this problem, THP-1 cells could be transiently transfected to overexpress LILRB3 prior to performing *S. pneumoniae* infection experiments. Another explanation for the data may be that THP-1 cells downregulated LILRB3 expression in response to *S. pneumoniae* infection. Indeed, preliminary co-localisation assays suggested surface LILRB3 was downregulated. Alternatively, it is possible that the PspC3.4 protein purified from the *E. coli* expression system had a different structural conformation and/or glycosylation pattern compared to PspC3.4 expressed on the surface of TIGR4. Therefore, future work is required to further investigate the interaction of PspC3.4 and LILRB3.

In conclusion, utilising bioinformatical and biochemical approaches potential novel interactions between LILRB3 and a subset of PspC proteins from *S. pneumoniae* were discovered. Additional work is required to demonstrate LILRB3 engagement with PspC3.4 expressed by *S. pneumoniae*, and to more broadly understand the depth of PspC subtypes that interact with LILRB3. For example, it would be interesting to investigate LILRB3 interaction with PspC7- and PspC4-expressing *S. pneumoniae* strains given the biochemical data supporting protein-protein interactions. It is also important for future studies to explore LILRB3 interaction using neutrophil and/or neutrophil-like cells that are known to highly express surface LILRB3 (Lewis Marffy and McCarthy 2020; Zhao et al. 2020). Elucidation of the functional consequences of LILRB3-PspC interaction, and whether this benefits host or pathogen, is necessary to fully understand the pathogenesis of *S. pneumoniae* and may aid development of novel therapeutics.

Supplementary figure 5.1

PspC subtype	Sequence
psp C1. 1	MFASKSERKVHYSIRKFSIGVASVAVASLFLGGVVHAEGVRSNNLTVTSSGQDISKKYADEVESHLSESILKDVKKNLKKVQHTQNV GLITKLSEIKKKYLDLKVNVLSAEELTSKTKETKEKLATFEQFKDRTLPTPEKKVAAEQKKVEEAKKKAEDQKEKDRRNYPTITYKT LELEIAESDVEVKKAELVLVKAKESQDEEKIKQAAEVESKQAEATRLKKIKTDREEAKRKADAKLKEAVEKNVATSEQDKPKRRA KRGVSGELATPDKKENDAKSSDSSVGEETLPSPSLNMANESQTEHRKDVDEYIKKMLSEIQLDRRKHTQNVNLNIKLSAIKTKYLYEL SVLKENSKEELTSKTKAELTAAFEQFKDRTLKPEKKVAAEKKVVEAKKKAQDQKEEDRRNYPTNTYKTLLELEIAESDVVKKEAELEL VKEEANESRNEEKIKQAKEKVESKKAATRLKIKTDRKKAEEEEAKRKAEESEKKAAEAKQKVDAAEYALEAKIAELEVEVQRLEKELK EIDESDSEDYLKEGLRAPLQSKLDTKKAKLSKLEELSDKIDELDAEIAKLEVQLKDAEGNNNNVEAYFKEGLEKTTAEKKAELEKAEADL KKAVDEPETPAPAPQPAPAPEKPAEKPPAPAPEKPAPAPEKPAPAPEKPAPAPEKPAPAPEKPAPTPTETPKTGWKQENGMMWYFYNN TDGSMATGWLQNNGSWYYLNSNGAMATGWLQNNGSWYYLNSNGAMATGWLQYNGSWYYLNGANGMATGWLQYNGS WYYLNGANGMATGWFQYNGSWYYLNGANGMATGWFQYNGSWYYLNGANGMATGWLQYNGSWYYLNSNGAMVTGWL QNNGSWYYLNGANGSMATDWVKDGDWTWYLEASGAMKASQWFKVSDNWYYVNGSGALAVNTTVDSYRVNPNNGEWWN
psp C1. 2	MFASKSERKVHYSIRKFSIGVASVAVASLFLGGVVHAEGVRSNNLTVTSSGQDISKKYADEVESHLSESILKDVKKNLKKVQHTQNV GLITKLSEIKKKYLDLKVNVLSAEELTSKTKETKEKLATFEQFKDRTLPTPEKKVAAEQKKVEEAKKKAEDQKEKDRRNYPTITYKT LELEIAESDVEVKKAELVLVKAKESQDEEKIKQAAEVESKQAEATRLKKIKTDREEAKRKADAKLKEAVEKNVATSEQDKPKRRA KRGVSGELATPDKKENDAKSSDSSVGEETLPSPSLNMANESQTEHRKDVDEYIKKMLSEIQLDGRKHTPNVNLNIKLSAIKTKYLYEL SVLKENSKEELTSKTKAELTAAFEQFKDRTLKPEKKVAAEKKVVEAKKKAQDQKEEDRRNYPTNTYKTLLELEIAESDVVKKEAELEL VKEEANESRNEEKIKQAKEKVESKKAATRLKIKTDRKKAEEEEAKRKAEESEKKAAEAKQKVDAAEYALEAKIAELEVEVQRLEKELK EIDESDSEDYLKEGLRAPLQSKLDTKKAKLSKLEELSDKIDELDAEIAKLEVQLKDAEGNNNNVEAYFKEGLEKTTAEKKAELEKAEADL KKAVDEPETPAPAPQPAPAPEKPAEKPPAPAPEKPAPAPEKPAPAPEKPAPAPEKPAPAPEKPAPAPEKPAPAPEKPAPAPKPT PETRLETRKRYVGTSTILMGSMATGWLQNNGSWYYLNSNGVMXTGWXPNNNGHGLPXRNGVWGRLPVXWSRXYXNVIMGFXE GWSINGXGLPXVXGLGRWSXKARXSQRMGLEVAH----- ----- ----- ----- -----
psp C2. 1	MFASKSERKVHYSIRKFSIGVASVVASLVMGSVVHATENEGSTQAATFSNMANKSQTEQGEINIERDKAKTAVSEYKEKKVSEIYT KLERDRHKDVTDLVNKLQEIKNYLNKIVQSTSTEIQGLITTSRSLKDEAVSKYKAPSSSSSSGSSTKPEASDTAKPNKPTLEKKVA EAEKKVEEAKKKAQDQKEEDYRNYPTITYKTLLELEIAESDVEVKKAELVLKEEAKPRNEEKVKQAKAKVESEETEATRLKIKTDRK KAEAAEAKRKAEEEDKVEKPAEQQAEEYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMMWYFYNTDGSMTGWL QNNGSWYYLNSNGAMATGWLQNNGSWYYLNGANGSMATGWLQNNGSWYYLNGANGSMATGWLQYNGSWYYLNGANGDM ATGWLQYNGSWYYLNGANGMATGWLQYNGSWYYLNGANGMATGWVKDGDWTWYLEASGAMKASQWFKVSDKWYYVN GSGALAVNTTVDGYGVNANGWVN----- ----- ----- ----- -----
psp C2. 2	MFASKSERKVHYSIRKFSVGVASVVASLVMGSVVHATENEGATQVPTSSNRANESQAEQGEQPKKLDSEDKARKEVEEYVKKI VGESYAKSTKKRHTITVALVNELNNIKNEYLNKIVESTESQLQILMMESRSKVDEAVSKFEKSSSSSSSSDSTKPEASDTAKPNKPT EPGEKVAEAKKKVEEAKKKAQDQKEEDRRNYPTITYKTLLELEIAESDVEVKKAELVLVKKANEPREDEQKIKQAAEVESKQAEATRL KKIKTDREEAAEEAKRRDAKEQGPKPKGRAKRGVPGELATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAAEKKVVEEAKKKAED QKEEDRRNYPTITYKTLLELEIAESDVEVKKAELVLKEEAKPRNEEKVKQAKAEVESKKAATRLKIKTDRKKAEEAAEAKRKAEEEDK VKEKPAEQPPAPAPKAEPAPAPKPNPAEQPKAEKPADQQAEEYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQEN GMWYFYNTDGSMTGWLQNNGSWYYLNSNGAMATGWLQNNGSWYYLNGANGSMATGWLQNNGSWYYLNGANGSMATG WLQYNGSWYYLNGANGMATGWLQYNGSWYYLNGANGMATGWVKDGDWTWYLEASGAMKASQWFKVSDKWYYVNGSGA LAVNTTVDGYGVNANGWVN----- ----- ----- ----- -----

psp C2. 3	MFASKSERKVHYSIRKFSIGVASVVASLVMGVSVVHATEKEGATQVPTSSNRANESQAEQGEQPKKLDSEKDARKEVEEYVKKIV GESYAKSTKKRHTITVALVNELNNIKNEYLNKIVESTSESQQLIMMESRSKVDEAVSKFEKDSSSSSSSSSTKPEASDTAKPNKPT PGEKVAEAKKKVEEAEKKAKDQKEEDRRNYPTITYKTLELEIAESDVEVKKAELELVKVKANEPDEEKIKQAEAEVESKQAEATRLK KIKTDREKAEGEAKRRADAKEQDESSKRRKSRVGRDLGEQATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVAEAKKK AEDQKEEDRRNYPTNTYKTLELEIAESDVEVKKAELELVKEEAKPRNEEKVKQAKAEVESKKAETRLKIKTDREKAAEEAAKRAA EEDKVKEKPAEQQPAPAPQPEKPAPKPNPAEQPKAEKPADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQE NGMWYFYNTDGSMTATGWLQNNGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWLQNNGSWYYLNANGDMAT GWLQYNGSWYYLNSNGAMVTGWLQYNGSWYYLNSNGAMVTGWVKDGDWYYLEASGAMKASQWFKVSDKWYYVNGSG ALAVNTTVDGYGVNANGEWVN----- -----
psp C2. 4	MFASKSERKVHYSIRKFSIGVASVVASLVMGVSVVHATEKEGATQVPTSSNRANESQAEQGEQPKKLDSEKDARKEVEEYVKKIV GESYAKSTKKRHTITVALVNELNNIKNEYLNKIVESTSESQQLIMMESRSKVDEAVSKFEKDSSSSSSSSSTKPEASDTAKPNKPT PGEKVAEAKKKVEEAEKKAKDQKEEDRRNYPTITYKTLELEIAESDVEVKKAELELVKVKANEPDEEKIKQAEAEVESKQAEATRLK KIKTDREAAEEAAKRRADAKEQKPKRRKGRKRGVPGQATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAKK KAEDQKEEDRRNYPTNTYKTLELEIAESDVEVKKAELELVKEEAKPRNEEKVKQAKAEVESKKAETRLKIKTDREKAAEEAAKRAA AEDKVKEKPAEQQPAPAPKAEKPAPKPNPAEQPKAEKPADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWK QENGWYFYNTDGSMTATGWLQNNGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWLQYNGSWYYLNSNGAM VTGWLQYNGSWYYLNSNGAMVTGWLQNNGSWYYLNANGDMATGWLQYNGSWYYLNSNGAMVTGWVKDGDWYYLEASGAMKASQWFKVSDKWYYVNG SGALAVNTTVDGYGVNANGEWVN----- -----
psp C3. 1	MFASKSERKVHYSIRKFSIGVASVAVASLVMGVSVVHATENEGSTQAATSSNMAKTEHRKAAKQVVDEYIEKMLREIQLDRRKHTQ NVALNIKLSAIKTKYLRELVLEEKSKDELPEIKAKLDAAFEKFKKDTLKPGEKVAEAKKKVEEAKKKAEDQKEEDRRNYPTNTYKT LELEIAEFDVKVKEAELELVKEEAKESRNEGTIKQAEKVESKKAETRLNIKTDRKKAEEAAKRAKADAKLEANVATSDQGPKGRA KRGVPGELATPDKKENDAKSSDSSVGEETLPSSSLKSGKKVAEAEKKVEEAEKKAKDQKEEDRRNYPTNTYKTLDLEIAESDVKVKE AELELVKEEAKPRDEEKIKQAKAKVESKKAETRLNIKTDRKKAEEAAKRAAEEEDKVKEKPAEQQPAPATQPEKPAPKPEKPA EQPKAEKTDQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGWYFYNTDGSMTATGWLQNNGSWYYLN ANGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYLNANGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGS WYYLNANGDMATGWLQNNGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWLQYNGS WYYLNSNGAMATGWLQYNGS FKVSDKWYYVNGSGALAVNTTVDGYGVNANGEWVN----- -----
psp C3. 2	MFASKSERKVHYSIRKFSIGVASVAVASLVMGVSVVHATENEGSTQAATSSNMAKTEHRKAAKQVVDEYIEKMLREIQLDRRKHTQ NVALNIKLSAIKTKYLRELVLEEKSKDELPEIKAKLDAAFEKFKKDTLKPGEKVAEAKKKVEEAKKKAEDQKEEDRRNYPTNTYKT LELEIAEFDVKVKEAELELVKEEAKESRNEGTIKQAEKVESKKAETRLNIKTDRKKAEEAAKRAAEEEDKVKEKPAEQQPAPATQ PEKPAPKPEKPAEQPKAEKTDQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGWYFYNTDGSMTATGWL QNNGSWYYLNANGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYLNANGAMATGWLQYNGSWYYLNSNGAM ATGWLQYNGSWYYLNANGDMATGWLQNNGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWLQYNGS SGAMKASQWFKVSDKWYYVHGSSALAVNTTVDGYGVNANGEWVN----- ----- -----
psp C3. 4 TIG R4	MFASKSERKVHYSIRKFSIGVASVVASLVMGVSVVHATENEGSTQAATSSNMAKTEHRKAAKQVVDEYIEKMLREIQLDRRKHTQ VGESYAKSTKKRHTITVALVNELNNIKNEYLNKIVESTSESQQLIMMESRSKVDEAVSKFEKDSSSSSSSSSTKPEASDTAKPNKPT EPGEKVAEAKKKVEEAEKKAKDQKEEDRRNYPTITYKTLELEIAESDVEVKKAELELVKVKANEPDEEKIKQAEAEVESKQAEATRL KKIKTDREAAEEAAKRRADAKEQKPKGRKRGVPGELATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAKKKAED QKEEDRRNYPTNTYKTLELEIAESDVEVKKAELELVKEEAKPRNEEKVKQAKAEVESKKAETRLKIKTDREKAAEEAAKRAAEEED KVKEKPAEQQPAPAPKAEKPAPKPNPAEQPKAEKPADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQE NGMWYFYNTDGSMTATGWLQNNGSWYYLNSNGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYLNANGSMAT GWLQYNGSWYYLNANGSMATGWLQYNGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWLQYNGS ALAVNTTVDGYGVNANGEWVN----- -----
psp C3. 3	MFASKSERKVHYSIRKFSIGVASVAVASLVMGVSVVHATENEGSTQAATSSNMAKTEHRKAAKQVVDEYIEKMLREIQLDRRKHTQ NVALNIKLSAIKTKYLRELVLEEKSKDELPEIKAKLDAAFEKFKKDTLKPGEKVAEAKKKVEEAKKKAEDQKEEDRRNYPTNTYKT LELEIAEFDVKVKEAELELVKEEAKESRNEGTIKQAEKVESKKAETRLNIKTDRKKAEEAAKRAKADAKLEANVATSDQGPKGRA KRGVPGELATPDKKENDAKSSDSSVGEETLPSSSLKSGKKVAEAEKKVEEAEKKAKDQKEEDRRNYPTNTYKTLDLEIAESDVKVKE AELELVKEEAKPRDEEKIKQAKAKVESKKAETRLNIKTDRKKAEEAAKRAAEEEDKVKEKPAEQQPAPATQPEKPAPKPEKPA

	EQPKAEKTDQQAEDYARRSEEEYNRLIQQQPPKTEKPAQPFTPKTGWKQENGMMWYFYNTDGSMTATGWLQYNGSWYYLNA NGDMATGWVKDGDWTWYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYGVNANGEWVN----- ----- -----
psp C3. 5	MFASKKERVHYSIRKFSIGVASVVASLFMGSVVHATERGGSTPVATSSNKANKSQTEHMKAAEQVDEYVNMQLDRKHTQ NLALNIKLSAIKTKYLRELVLEKKSKEELTSKTKKEIDAAFEQFKKDTLKPGEKVEEAQKKVEEAEEKAKDQKEEDRRNYPTITYKTL ELEIAESDVEVKKAELELVKVKAKGSRNEEKIKKAKAEVESKKAETKLEIKTERKKAEEAKRKAEEVEVKDKLKKRTKRGALGEP ATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVAEAEKKAKDQKEEDRRNYPTNTYKTLLEIAESDVVKKEAELELVKEE AKEPQNEEKIKQAKAKVESKKAETRLKIKTDREKKAEEAKRKAEEEDKVKEKPAEQPQAPAPAEKPAEQPKAEQPKAEKPAD QQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMMWYFYNTDGSMTATGWLQYNGSWYYLNSNGAMATGW LQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATG ATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATG NGDMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNA NGDMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNA NGEWVN----- -----
psp C3. 6	MFASKSERVHYSIRKFSIGVASVAVASLFMGSVVHATEKEVTTQVATSSNRANESQAGHRKAAEQFDEYIKTMIQLDRRKHTQN FALNIKLSRIKTEYLRKLVLEEKSKAELPSETKKEIDAAFEQFKKDTNRTKKTVAEAEKKVEEAKKKAKAQKEEDHRNYPTNTYKTL EIAESDVEVKKAELELVKEEAKESRDDEKIKQAEAKVESKKAETRLKIKTDREKKAEEAKRKAEEAKLKEAVEKNVATSEQGPKGR AKRGVPGEQATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVAEAEKKAKAQKEEDRRNYPTNTYKTLLEIAESDVVKV EAELELVKEEAKESRNEEKVNQAKAKVESKKAETRLKIKTDREKKAEEAKRKAEEEDKVKEKPAEQPQAPAPAEKPAEQPKTEEPEN APAPKPEKPAEQPKAEKPDDQQAEDYARRSEEEYNRLTQQQPKPEQAPAPKIGWKQENGMMWYFYNTDGSMTATGWLQY NGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATG WLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATG DMATGWVKDGDWTWYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYGVNANGEWVN----- -----
psp C3. 7	MFASKNERVHYSIRKFSIGVASVAVASLFMGSVVHATEAGGNYPSSPCSSSEANKSQTEHMKAAEQGKQHREIDLDRNKAKAI DEYIEKMLSEIQLDRKHTQNFAFNILKSRIKTEYLYGLSVLEKSEAEALPSKAEKIKELTAAFEHFKKDTLRPGEKVAEAKKVEE AEKKAKDQKEEDRRNYPTITYKTLLEIAESDVEVKKAELELVKEEANKPRNEQKVQAKAKVESKQAEATRLKIKTDREAEAEAK RRAEAKLKEAVEKNATSEQGPKGRAKRGALGEPATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAEEKAKDQK EEDRRNYPTNTYKTLDEIAESDVVKKEAELELVKEEAKPRDEEKIKQAEKVESKKAETRLKIKTDREKKAEEAKRKAEEEDKVKE KPAEQPQAPAPAEKPAEKPAEHPKAENRADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMMWY FYNTDGSMTATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQY GWSYYLNRNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQY WFKVSDKWYYVNGSGALAVNTTVDGYGVNANGEWVN----- -----
psp C3. 8	MFASKSERVHYSIRKFSIGVASVAVASLFMGSVVHATEKEVTTQVATSSNRANESQAGHRKAAEQFDEYIKTMIQLDRRKHTQN FALNIKLSRIKTEYLRKLVLEEKSKAELPSETKKEIDAAFEQFKKDTNRTKKTVAEAEKKVEEAKKKAKAQKEEDHRNYPTNTYKTL EIAESDVEVKKAELELVKEEAKESRDDEKIKQAEAKVESKKAETRLKIKTDREKKAEEAKRKAEEAKLKEAVEKNVATSEQDKPKGR RKRGVPGEQATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVAEAEKKAKAQKEEDRRNYPTNTYKTLLEIAESDVVKV EAELELVKEEAKESRNEEKVNQAKAKVESKKAETRLKIKTDREKKAEEAKRKAEEEDKVKEKPAEQPQAPAPAEKPAEQPKTEEPEN APAPKPEKPAEQPKAEKPDDQQAEDYARRSEEEYNRLTQQQPKPEQAPAPKIGWKQENGMMWYFYNTDGSMTATGWLQY NGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQY GWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATG DMATGWVKDGDWTWYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYGVNANGEWVN----- -----
psp C3. 9	MFASKNERVHYSIRKFSIGVASVAVASLFMGSVVHATEKVVTTQVATSSNKANKSQTEYMKAAKQVDEYITKKLQLDRREHIQN VGLLTKLGVIKTEYLRHLSVSKEKSEAEALPSEIKAKLDAAFELFKKDTLPTEPGKKVAEAEKKVEEAKKKAEKQKEEDRRNYPTNTYKTL ELDIAESDVEVKKAELELVKEEAKESRDEKINQAKAKVENKKAETRLKIKTDREKKAEEAKRKAEEAKLQAEANVATSEQDKSKRRA NREVLGELATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAKKKAEKQKEEDRRNYPTNTYKTLLEIAESDVEVKK EAELELVKEEAKPRNEEKVKQAKAEVESKKAETRLKIKTDREKKAEEAKRKAEEEDKVKEKPAEQPQAPAPKAAAPKPKENPAEQ PKAEKPADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMMWYFYNTDGSMTATGWLQYNGSWYYLNSN GAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYY

	LNANGDMATGWVKDGDWYYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYGVNANGEWVN----- ----- -----
psp C3. 10	MFASKNERKVHYSIRKFSIGVASVAVASLFMGSVVHATEKEVTTQVPTSSNRANKSQTEHMRRAAKQVDEYIKKKQLDRRKHTQN VGLLTGLGVIKTEYLHGLSVSKEKSEALPSEVKAKLDAAFEQFKKDTLPTGPKKVAEAEKKVEEAKKKAEDQKEKDLRNYPTNTYK TLELDIAESDVEVKKAELELVKGGGYKPRDEQKVNQAKAKVESKQAEATRLKKIKTDREEAEAAKRRAEAKLKEAVEKNTATSEQG KPKRRGKRGALGEQATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAKKAKDQKEEDRRNYPTNTYKLELEIAES DVVKKEAELELVKEEAKESRNEEKIKQVKAKVESKQAEATRLNIKTDRKKAEEEEAKRKAAEEDKVKEKPAEQPPAPAPQPEKPT EPEEPAPAPKPENPAEQPKAEKPADQQAEDYARRSEEEYNRLTQQQPPKIEKVPVAPHPEPQAPAPKTGWKQENGMMWYFYNT DGSMATGWLQNNGSWYYLNSNGAMATGWLQNNGSWYYLNGANGDMATGWLQYNGSWYYLNGANGSMATGWLQYNGSW YYLNGANGSMATGWLQNNGSWYYLNGANGSMATDWVKDGDWYYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDG YGVNANGEWVN----- -----
psp C3. 11	MFASKSERKVHYSIRKFSIGVASVAVASLFMGSVVHATEKEVTTQVPTYSNMAKTEHRKAQVDEYIEKMLREIQLDRRKHTQ NFAFNMKLSAIKTEYLYGLKEKSEALPSEVKAKLDAAFEQFKKDTLKGKVAEAEKKVAEAEKKAKAKQKEEDRRNYPTNTYKLEL EIAESDVEVKKAELELLKEEAKTRNEDTINQAKAKVESKQAEATKLEIKTDRKKAEEEEAKRKAGAEEDKVVDKLRRAKRAVPGEP TPDKKENDAKSSDSSVGEETLPSPSLKSGKRSKGAEKRCRAEKKAKDQKEEDRRNYPTNTYKTLDEIAESDVVKKEAELELVKEEAKG SRNEEKINQAKAEVESKQAEATRLKIKTDRKKAEEEEAKRKAAEEDKVKEKPAEQPPAPDPQPEKPTPEENPAPAPKPEKPAEQ KAEKTDQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMMWYFYNTDGSMATGWLQNNGSWYYLNG AMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNGANGDMAIGVWQYNGSWYYL NANGDMATGWLQYNGSWYYLNGANGSMATGWVKDGDWYYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYG VNANGEWVN----- -----
psp C3. 12	MFASKNERKVHYSIRKFSIGVASVAVASLFMGSVVHATEKEVTTQVATSSNANKSQTEHMKAAKQVDEYIEKMLSEIQLDRRKH TQNVGLLKLGAIKTEYLRGLSVSKEKSTAEALPSEIKELTAAFEQFKKDTLKGKVAEAEKKVEEAKKVAEAEKKAKDQKEEDRR NYPTNTYKLELEIAEFQVVKKAELELVKEEAREPRDEGTIKQAKAEVESKQAEATRLKIKTDRKKAEEEEAKRKAAEEDKVKEKPA EQQQPAPAPQPEKPAKPENPAEQPKAEKPADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMMWYFYNT TDGSMATGWLQNNGSWYYLNSNGAMATGWLQNNGSWYYLNGANGSMATGWLQNNGSWYYLNGANGSMATGWLQYNGS WYYLNGANGSMATGWLQNNGSWYYLNGANGDMATGWVKDGDWYYLEASGAMKASQWFKVSDKWYYANGSGALAVNTTV DGYGVNANGEWVN----- ----- -----
psp C3. 13	MFASKSERKVHYSIRKFSIGVASVVASLFMGSVVHATENEGSTQAATSSNMAKTEHMKAAKQVDEYIKKKQLDRRKHTQNVG LLTKLGVKTEYLHGLSVSKEKSEALPSEIKAKLDAAFEQFKKDTLPTGPKKVAEAEKKVEEAKKKAEDQKEKDLRNYPTNTYKLEL DIAESDVEVKKAELELVKEEAKESRDEKINQAKAKVESEQAEATRLKKIKTDREKAEAAKRRADAKEQDESKRRKSRVKGDLGE PATPDKKENDAKSSDSGVGEETLPSPSLKPGKKVAEAEKKVEEAKKAKDQKEEDHRNYPTITYKLELEIAESDVEVKKAELELVKEE AKGSRNEEKVKQAKAEVESKQAEATRLKIKTDRKKAEEEEAKRKAAEEDKVKEKPAEQPPAPAPQPEKPAKPEKPAEQPKAE KPADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMMWYFYNTDGSMATGWLQNNGSWYYLNSNGAM ATGWLQNNGSWYYLNGANGSMATGWLQYNGSWYYLNGANGSMATGWLQYNGSWYYLNGANGDMATGWQYNGSWYYLNA NGDMATGWLQYNGSWYYLNSNSAMVTGWLQYNGSWYYLNGANGSMATDWVKDGDWYYLEASGAMKASQWFKVSDKWY YVNGSGALAVNTTVDYRVNANGEWVN----- ----- -----
psp C4. 1	MFASKSERKVHYSIRKFSIGVASVVASLFVGGVHAAEVRNGNLTVTSSGDEVESHYQSILEKVRKSLEKDRHTQNVDLIKKLQDI KRTYLYNLKEKPEALTSKTNKELDAAFEKFKKEPCLKLAEEAEKKAKDQKEEDHRNYPTNTYKLTIELEIAEAEVGVAKAELELAQAQ VQIPQDTEKINAAKAKVEAAKSNVKKLEIKSDIEKTYLYKLDNSTKETPKSRVRRNSPQVGDSRELKETIDKAKETLSTYMTVRLTKL DPSVFWFADLLMDAKKVVEEYTKLEDASDKSVEDLRKEAEGKIESLIVTHQNRKENQAPQPGGQAGGSMVPPVPTQTPPS TSQSPGQKATEAEKKKLQDLIRQFQALNKLDDETKTVPDGAKTGEAGKAYNETRTYAKEVVDKSKKLSQTAVTMDLAMQLT KLNDAMSKLKEAKAKLVPEVKPQPENPEPKPQPEGEKPSVPDINQEKEKAKLAIATYMSKILDDIKKHHLKKEKHQIVALIKDLKDL RKQALSEIDNVNTKVEIENTVHKVFADMDTVTKFQKGLIQNTPVPEAQRAQRYQRFQIHQKAPDTPQVPEAPKSPEVPKVP APKAPDTPQVPEAPKSPEVPKVSPTKAPDTPQVPEAPKSPEVPKVPPEAPKAPDTPQVPEAPKSPEVPKVPDTPKAPDTPQVPEA PKAPDTPQIPEAPAPETPAPAPEAPKTGWKQENGMMWKG----- ----- -----

psp C6. 2	MFASKSERKVHYSIRKFSVGASVVASLFLGGVVHAEGVRSGNNSTVTSSGQDISKKYADEVESHLQSIKLDVNKNLKKVQHTQN ADFNKKLSKIKTKYLYELSVLEEKSEAELTSKTKETKEELTAAFEQFKKDTLSTEPEKKVAEAKKKVEEAKKKAEDQKEKDRRNYPTITY KTLELEIAESDVEVKKAELELVKVKANEPDRDEEKIKQAEAKVESKQAEATRLNIKTDRQAEAAEKVKDEPKKRTKRGVLGEPATPD KKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAKKKAEDQKEKDRRNYPTITYKTLELEIAESDVEVKKAELELVKEEAKPR NEEKVKQAKAEVESKQAEATRLKIKTDRKKAEEEEAKRKAEEEDKVKEKPAEQPQPAPAPQPEKPAPKPEKPAPAPKPNPAEQPK AEKPAAQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMWYFYNTDGSMTATGWLQNNGSWYYLNANGA MATGWLQNNGSWYYLNSNGAMATGWLQNNGSWYYLNSNGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYL NANGDMATGWVKDGDWTWYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYGVNANGGEWVN----- -----
psp C6. 3	MFASKSERKVHYSIRKFSVGASVAVASLFLGGVHAEGVRIGNNSTVSSSGDEVESHLSILKLDVNKNLKKVQHTQNVGLLTKLSE IKRKYLYELKVNGLLEEKSKAELTSKTKKELTAAFEQFKKDTLSTELEKKVAEAQKKVAEAEKKAKAQKEEDHRNYPTNTYKTLELEIAES DVEVKKADLKLKEEAKESRDEGTIKQAEAKVESKQAEATKLEIKTDRKAEAAEKRRAGANCRKLMLRLQGQDKPKGRAKRAVL GEPATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAKKKAEDQKEEDRRNYPTNTYKTLELEIAESDVKVKEAELELV KEEAKPRDEEKIKQAKAEVESKQAEATRLKIKTDRKKAEEEEAKRKAEEEDKVKEKPAEQPQPAPAPQPEKPAPKPEKPAEQPKAE KPADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMWYFYNTDGSMTATGWLQNNGSWYYLNANGAM ATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYLNANGAMATGWLQYNGSWYYLNSNGAMATGWLQYNGSWYYLNA NGDMATGWVKDGDWTWYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYGVNANGGEWAN----- -----
psp C6. 4	MFASKSERKVHYSIRKFSVGASVVASLFLGRVVHAEGVRSGDTPKVTSSGQDISKKYADEVKSHLEKILSEIQLDKRKHTQNLAF NKKLSRIQTEYFYLKKLKAELTSKTKKEELTSKTEELTSKTKKELDAAFEQFKKDTLSTEPEKKVAEAQKKVAEAEKKKAEDQKEEDRRN YPTITYKTLELEIAEFDVVKKAELELVKVKANEPDRDEQKIKQAEAEVESKQAEATRLKKIKTDREEAEAAEKRRADAKEQGGPKPAE QPQPAPAPQPEKPAPAPAPKPNPAEQPKAEKPADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQEN GMWYFYNTDGSMTATGWLQNNGSWYYLNSNGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYLNANGSMATG WLQYNGSWYYLNANGSMATGWLQNNGSWYYLNANGDMATGWVKDGDWTWYLEASGAMKASQWFKVSDKWYYVNGSGA LAVNTTVDGYGVNANGGEWVN----- -----
psp C6. 5/9 .2	MFASKSERKVHYSIRKFSIGVASVVASLFLGGVVHAEEVGGKNTPTDTSQGDISKKYADEVKSHLEKILSEIQTQLDRKRHTETVA LINELQGIKTYLYNLNLVLEKSKVELPSKIKAKLDVAFDQFKKDTLKPGEKVAEAQKKVAEAEKKKAEDQKEEDRRNYPTNTYKTLEL DIAESDVKVKEAELETSKRGAKPRNEEKIKKAKAKVESEKAEAIRLEEIKTDREEAKRKADAKLKEAVEKNAANSEQGEPPRRVVRGV LGEPATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAKKKAEDQEEEDRRNYPTNTYKTLELDIAESDVKVKEAELEL VNEEAKPRNEEKIKKAKAKVESEKAEATRLKIKTDRKKAEEEEAKRKAEEEDKVKEKPAEQPQPAPAPQPEKPAEENPAPAPKPEK PADQPKAEKPADQQAEDYARRSEEEYNRLTQQQPPKPEQPAPAPKTGWKQENGMWYFYNTDGSMTATGWLQNNGSWYYL NSNGAMATGWLQYNGSWYYLNANGDMATGWFQYNGSWYYLNANGDMATGWFQYNGSWYYLNANGDMATGWLQYNG SWYYLNSNGAMVTGWLQNNGSWYYLNANGSMATDWWKGDGTWYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVD DSYRVNANGGEWVN----- -----
psp C6. 6	MFASKSERKVHYSIRKFSVGASVVASLFLGGVVHAEGVRSGDTPKVTSSGQDISKKYADEVKSHLEKILSEIQLDKRKHTQNLAF NKKLSRIQTEYFYLKKLKAELTSKTKKEELTSKTEELTSKTKKELDAAFEQFKKDTLSTEPEKKVAEAEKKDAEAKKKAEDQKEEDRRNY PTNTYKTLELEIAEFDVVKKAELELLKEKAKESRDEEKIKQAEAEVESKQAEATRLNIKTDRKKAEEEEAKRKADAKLKEANVATSDQ GKPKGRAKRGVPGELATPDKKENDAKSSDSSVGEETLPSSSLKSGKKVAEAEKKVEEAEKKKAEDQKEEDRRNYPTNTYKTLELEIAE SDVKVKKAELELVKEEAKPRDEEKIKQAKAEVESKQAEATRLKIKTDRKKAEEEEAKRKAEEEDKVKEKPAEQPQPAPAPQPEKPA PKPKTSSDKPENPAEQPKAEKPADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMWYFYNTDGSMT GWLQNNGSWYYLNSNGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYLNANGSMATGWLQYNGSWYYLNANG SMATGWLQYNGSWYYLNANGDMATGWVKDGDWTWYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYGVNAN GEWVN----- -----
psp C6. 7	MFASKSERKVHYSIRKFSIGVASVVASLFLGGVVHAEGVRSENNPTVTSSGQDISKKYADEVKSHLEKILSEIQTNLDRSKHIKTVNL INKLQDIKRTYLYELNVLEDKSKAELPSKIKAELEDAFEQFKKDTLPTEPGKKVAEAKKKVEEAEKKAKAQKEEDYRNYPTITYKTLELEI AESDVKVKEAELELVKKEADESRNEGTINQAKAKVESEKQAEATRLKKIKTDRKAEAAEKRRADAKEQDESKRRKSRVVRKRGDLGEP TPDKKNDNAKSSDSSVGEETLPSPSLKPGKKVADAEEKVEEAEKKKAEDQKEEDHRNYPTITYKTLELEIAESDVEVKKAELELVKEEA KGSRNEEKVKQAKAEVESKQAEATRLKIKTDRKKAEEEEAKRKAEEEDKVKEKPAEQPQPAPAPQPEKPAPAPKPNPAEQPKAEK RADQQAEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMWYFYNTDGSMTATGWLQYNGSWYYLNANGAIAT

	GWFQYNGSWYYLNANGSMATGWLQYNGSWYYLNANGSMATGWLQYNGSWYYLNANGDMATGWFQYNGSWYYLNANG DMATGWLQYNGSWYYLNSNGAMVTGWLQYNGSWYYLNANGSMATDWVKDGDWYYLEASGAMKASQWFKVSDKWYYV NGSGALAVNTTVDSYRVNANGWVN----- -----
psp C6. 8/9 .3	MFASKNERKVHYSIRKFSIGVASVAVASLFLGGVHAEGVRIGNNSMVTISIGDEVESHLSILKDVNKNLQKVQHTQNVGLLSKLS EIKRKYLKLVNGLLEEKSKAELTSKTKKELTAAFEQFKKDTLSTELEKKVAEAQKKVAEAEKKAKAQKEEDHRNYPTNTYKTLLEIAE SDVGVKAELELLKEGAKESRDEGPIKQAGAKVESKKAEEATKLEIKITDREKAEAEAKRRADALQEANVATSGQDESKRRAKRGVP GELATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAKKKAEDQKEEDRRNYPTNTYKTLLEIAESDVEVKAELELV KEEAKEPRNEEKVKQAKAEVESKKAEEATRLEIKITDRKKAEEAKRKAEEEDKVKEKVPAPQPAPAPQPEKPAEETPAPAPKPEKPT EQPKAEKPDQQAEEEDYARRSEEEYNRLTQQQPPKPEQPAPAPKIGWKQENGWYFYNTDGSMTGWLQNNGSWYYLNAN GAMATGWLQNNGSWYYLNANGSMATGWLQYNGSWYYLNSNGAMATGWLQNNGSWYYLNANGSMATGWLQYNGSWYY LNSNGAMVTGWLQNNGSWYYLNANGSMATDWVKDGDWYYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYG VNANGWVN----- -----
psp C6. 9	MFASKSERKVHYSIRKFSIGVASVAVASLFLGGVHAEEVGGKNTPTDTSQGDISKKYADEVESHLKILSEIQTQLDRKHTETVA LINELQGIKTYLYNLNLVLEKSELPSKIKAKLDVAFDQFKKDTLKPGEKVAEAQKKVAEAKKKAEDQKEEDRRNYPTNTYKTLLEIDIA ESDVVKAELETSKRGAKPRNEEKIKKAKAVESEKAEAIRLEEIKTDREEAKRKADAKLKEAVEKNAANSEQGEPKRRVKRGVLGE PATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKKAEDQEEEDRRNYPTNTYKTLLEIDIAESDVVKAELELVNEEAKPRN EEKIKKAKAVESEKAEATRLEIKITDRKKAEEAEAKRKAEEEDKVKEKPAEQPPAPAPQPEKPAEENPAPAPKPEKPADQPKAEK PADQQAEEEDYARRSEEEYNRLTQQQPPKPEQPAPAPKTGWKQENGWYFYNTDGSMTGWLQNNGSWYYLNSNGAMATG WLQYNGSWYYLNANGDMATGWFQYNGSWYYLNANGDMATGWFQYNGSWYYLNANGDMATGWLQYNGSWYYLNSNGA MVTGWLQNNGSWYYLNANGSMATDWVKDGDWYYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDSYRVNANG WVN----- -----
psp C6. 10	MFASKSERKVHYSIRKFSIGVASVAVASLFLGGVHAEGVRSWDTPKVTPSGQNISKHPDEVKSHLEKILSEIQLDRKHTQNLAF NKKLSRIQTEFYLYLKKLKAELTSKTEELTSKTEELTSKTKKELDAAFEQFKKDTLSTEPEKKVAEAQKKVAEAKKKAEDQKEEDRRN YPTNTYKTLLEIAEAFDVVKAELELLKEAKDTRDEEIKQAEAEVESKQAEATRLKIKITDREEAEAEAKRRADAKEKEAEAEPSD QGKPKGRAKRGVPGELATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKVEEAKKKAEDQKEEDRRNYPTNTYKTLLEIA ESDVEVKAELELVKEEAKEPRNEEKVKQAKAEVESKKAEEATRLEIKITDRKKTEEEAKRKAEEEDKVKEKPAEQPPAPAPQPEK APKPENPAEQPKAEKPADQQAEEEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGWYFYNTDGSMTGWLQN NGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYLNANGSMAT GWLQYNGSWYYLNSNGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYLNANGSMATGWLQYNGSWYYLNANG SMATGWLQYNGSWYYLNANGDMATGWLKGDGTWYYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYGVNAN GEWVN----- -----
psp C6. 11	MFASKSERKVHYSIRKFSIGVASVAVASLFLGGVHAEGVGRNTSTVTSSGQDTSKKYADEVKSHYQSILEKVRKSLEKDRHTQNV GLITKLSEIKKLYLELVNVLLEEKSKAELPSKAKAELDAAFEQFKKEPELTKKVAEAQKKVEEAKKKAEDQKEEDFRNYPTNTYKTIE LEIAESDVVKAELELLKEEAKHRDEGTIKQVEEKVSEKAEATRLEEIKTERKKAEEAEAKRRADAKEQGKPKGRAKRGVPGEQAT PDKKENDAKSSDSSVGEETLPNPSLKPEKKVAEAEKKVEEAKKKAEDQKEEDHRNYPTITYKTLLEIAESDVEVKAELELVKEEAK GSRNEEKVKQAKAEVESKKAEEATRLEIKITDRKKAEEAKRKAEEEDKVKEKPAEQPPAPAPQPEKPAAPKPENPAEQPKAEKPA DQQAEEEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGWYFYNTDGSMTGWLQNNGSWYYLNANGSMATG WLQNNGSWYYLNANGAMATGWLQNNGSWYYLNANGDMATGWLQNNGSWYYLNANGSMVTGWLQNNGSWYYLNANG SMATDWVKDGDGTWYYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDSYRVNANGWVN----- -----
psp C6. 12	MFASKSERKVHYSIRKFSIGVASVAVASLFLGGVHAEDVGGRNTPVTSSGQDISKKYADEVKSHLEMLSGIQLNRRKHTQNFN FNLKLSKIQTLYLRELSVLKSSELTLETKKELSKTAEALDAAFEQFKKDTLKLGEKVAEAEKKVEEAEKKAKAQKEKDRNYPTNTYK TLELEIAESDVGVKAELELLKEAKTRNKDITINQAKAKVESKQAEATRLEIKITERKAEAEAKRKADAKLKEAVEKNAVTSQEGELK RRTKRAVPGEPATPDKKENDAKSSDSSVGEETLSSPSLKPGKKVAEAEKKVEEADKAKAQKEEDHRNYPTNTYKTLLEIAESDVE VKKAELELVKEEAKEPRNEEKVKQAKAEVESKKAEEATRLEIKITDRKKAEEAEAKRKAEEEDKVKEKPAEQPPAPAPQPEKPTTE PENPAPAPKPEKPADHPKAEKTDQQAEEEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGWYFYNTDGSMTG WLQNNGSWYYLNANGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWYYLNANGAMATGWLQYNGSWYYLNANG DMATGWLQYNGSWYYLNANGDMATGWLKGDGTWYYLEASGAMKASQWFKVSDKWYYVNGSGALAVNTTVDGYGVNAN

	GEWVN----- -----
psp C6. 13/ 9.4	MFASKSERKVHYSIRKFSIGVASVVASLFLGGVVHAEVGGKNTPTDTSQGDISKKYADEVESHLKILSEIQTQLDRKRHTETVA LINELQGIKKTLYNLNLVLEKSELPSKIKAKLDVAFDQFKDITLKPGEKVAEAKKVAEAKKKAEDQKEEDRRNYPTNTYKLELDIA ESDVKVKEAELETSKRGAKEPRNEEKIKKAKAKVESEKAEAIRLEEIKTDREEAKRKADAKLKEAVEKNAANSEQGEPKRRVKGVLGE PATPDKKENDAKSSDSSVGEETLPSPSLKPEKKVAEAEKKKAEDQEEEDRRNYPTNTYKLELDIAESDVKVKEAELELVNEEAKPRN EEKIKKAKAKVESEKAEATRLEIKITDRKKAEEEEAKRKAEEEDKVKEKPAEQPPAPAPQPEKPAEENPAPAPKPEKPADQPKAEK PADQQAEEEDYARRSEEEYNRLTQQQPPKPEQPAPAPKTGWKQENGMMWYFYNTDGSMTATGWLQNNGSWYYLNSNGAMATG WLQYNGSWYYLNANGDMATGWFQYNGSWYYLNANGDMATGWFQYNGSWYYLNANGDMATGWFQYNGSWYYLNANG DMATGWLQYNGSWYYLNSNGAMVTGWLQNNGSWYYLNANGSMATDWVKDGDWTWYLEASGAMKASQWFKVSDKWY VNGSGALAVNTTVDSYRVNANGWVN----- -----
psp C6. 14	MFASKSERKVHYSIRKFSVGVASVVASLFLGRVVHAEVGRSGDTPKVTSSGYADEVKSHLEKILSEIQLDKRKHTQNLAFNKKLSRI QTEFYFLKKLKAELAKTEVESEQAETTRLLKIKTDREEAELVKKIKTDREEAEDEVKDKPKKRTKRGALGEPATPDKKENDAKSSDS SVGEETLPSPSLKPEKKVAEAEKKVEEAEKKAKAKQKEEDRRNYPTNTYKLELEIAESDVKVKEAELELVKEEAKESRNEEKIKQVKAK VESKKAETRLENTSRQIRKKAEEEEAKRKAEEEDKVKETPAEQPPAPAPQPENPTEEPENPAPAPKPEKPAEQPKAEKPADQQ AEEEDYARRSEEEYNRLTQQQPPKTEKPAQPSTPKTGWKQENGMMWYFYNTDGSMTATGWLQNNGSWYYLNSNGAMATGWLQ NNGSWYYLNANGSMATGWLQNNGSWYYLNANGSMATGWLQYNGSWYYLNANGSMATGWLQYNGSWYYLNANGDMAT GWVKDGDWTWYLEASGAMKASQWFKVSDKWYVNGSGALAVNTTVDGYGVNANGWVN----- ----- ----- -----
psp C7. 1	MFKSNIYERKMCYSIRKFSIGVASVAVASLVMGVSVVHATENEGTTQAPTSSNRGNESQAEQRRELDLERDKVKKEVREYKEKKVKE LYSKSTKSRHKKTVDIVNKLQINNEYLNKIIQSTSTYEELQKLMMESQSEVDKAVSEFEKDLSSSSSSGSSTEPEASDTAKPNKPTL EKKVAEAAQKQVEEAEKKAKDQKEEDYRNYPTITYKLELEIAEFDVKVKEAELELVKAKESRDEKKIKQAEAEVESKQAEATRLLKI KTDRKKAEEEAELKEAVEKNAATSEQGKPKRRVKRRALGEQATPDKKDYFEKDFRPAFNKNRQMVAIQESLNKLDGETKTVPDGA KLTGEAGNAYNEVRDYAIKVVSSENKLLSQTAVTMDELAMQLTKLNDAMSKLREAKAKLVPEVKPQPENPEHQRPPTTPAPDTKPI PQPEGKKPSVPDINQEKEKAKLAVATYMSKILDDIQKHHLQKEKHRQIVALIKELDEFKKQALSEIDNVNTKVEIENTVHKIFADMD AVVTKFKKGLTQDTPKEPDNKKPSAPKPGMQSPQPEGKKPSVPAQPGTEDKKPSAPKPGMQSPQPEGKKPSVPAQPGTEDK KPSAPKPDMPSPQPEGKKPSVPAQPGTEDKKPSAPKPGMQSPQPEGKKPSVPAQPGTEDKKPSAPKPDMPSPQPEGKKPS VPAQPGTEDKKPSAPKPDMPSPQPEGKKPSVPEINQEKEKAKLAVATEKKLPSTGVASNVLVEIIGLLGLIGTSFIAMKRRK----- ----- -
psp C7. 2	MFKSNIYERKMCYSIRKFSIGVASVAVASLVMGVSVVHATENEGTTQAPTSSNRGNESQAEQRRELDLERDKVKKEVREYKEKKVKE LYSKSTKSRHKKTVDIVNKLQINNEYLNKIIQSTSTYEELQKLMMESQSEVDKAVSEFEKDLSSSSSSGSSTEPEASDTAKPNKPTL EKKVAEAAQKQVEEAEKKAKDQKEEDHRNYPTITYKLELEIAEFDVKVKEAELELVKAKESRDEKKIKQAEAEVESKQAEATRLLKI KTDRKKAEEEAELKEAVEKNAATSEQGKPKRRVKRGALGEQATPDKKDYFEKDFRPAFNKNRQMVAIQESLNKLDDETCTVPDGA AKLTGEAGNAYNEVRDYAIKVVSSENKLLSQTAVTMDELAMQLTKLNDAMSKLREAKAKLVPEVKPQPENPEHQRPPTTPAPDTK PIPQPEGKKPSVPDINQEKEKAKLAVATYMSKILDDIQKHHLQKEKHRQIVALIKELDEFKKQALSEIDNVNTKVEIENTVHKIFADM DAVVTKFKKGLTQNTPEPDNKKPSAPKPGMQSPQPEGKKPSVPAQPGTEDKKPSAPKPGMQSPQPEGKKPSVPAQPGTED KKPSAPKPGMQSPQPEGKKPSVPAQPGTEDKKPSAPKPDMPSPQPEGKKPSVPAQPGTEDKKPSAPKPDMPSPQPEGKKPS VPAQPGTEDKKPSAPKPDMPSPQPEGKKPSVPEINQEKEKAKLAVATEKKLPSTGVASNVLVEIIGLLGLIGTSFIAMKRRK----- ----- ----
psp C7. 3	MFKSNIYERKMCYSIRKFSIGVASVAVASLVMGVSVVHATENEGTTQAPTSSNRGNESQAEQRRELDLERDKVKKEVREYKEKKVKE LYSKSTKSRHKKTVDIVNKLQINNEYLNKIIQSTSTYEELQKLMMESQSEVDKAVSEFEKDLSSSSSSGSSTEPEASDTAKPNKPTL EKKVAEAKKQVEEAEKKAKDQKEEDRRNYPTITYKLELEIAESDVEVKAELELVKANESRDEKKIKQAEAEVESKQAEATRLLKI KTDRKKAEEEAELKEAVEKNAATSEQGKPKRRVKRRALGEQATPDKKDYFEKDFRPAFNKNRQMVAIQESLNKLDGETKTVPDGA KLTGEAGNAYNEVRDYAIKVVSSENKLLSQTAVTMDELAMQLTKLNDAMSKLREAKAKLVPEVKPQPENPEHQRPPTTPAPDTKPI PQPEGKKPSVPDINQEKEKAKLAVATYMSKILDDIQKHHLQKEKHRQIVALIKELDEFKKQALSEIDNVNTKVEIENTVHKIFADMD AVVTKFKKGLTQDTPKEPDNKKPSAPKPGMQSPQPEGKKPSVPAQPGTEDKKPSAPKPDMPSPQPEGKKPSVPAQPGTEDK KPSAPKPDMPSPQPEGKKPSVPAQPGTEDKKPSAPKPGMQSPQPEGKKPSVPAQPGTEDKKPSAPKPDMPSPQPEGKKPS VPAQPGTEDKKPSAPKPDMPSPQPEGKKPSVPEINQEKEKAKLAVATEKKLPSTGVASNVLVEIIGLLGLIGTSFIAMKRRK----- -----

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psp C7. 4	MFKSNYERKMCYSIRKFSIGVASVAVASLVMGVSVVHATENEGTTQAPTSSNRGNESQAEQRRELDLERDKVKKEVREYKEKKVKE LYSKSTKSRHKKTVDIVNKLQNNINNEYLNKIIQSTSTYEELQKLMMESQSEVDKAVSEFEKDLSSSSSSGSSTEPEASDTAKPNKPTL EKKVAEAQKQVEEAEEKAKDQKEEDHRNYPTITYKTLEIEAEFDVEVKEAELELVKVKAKESRDEKKIKQAEAEVESKQAEATRLKKI KTDRKKAEEEEAKLKEAVEKNAATSEQGKPKRRVKRRALGEQATPDKKDYFEKDFRPAFNKNRQMVAIQESLNKLDGETKTVPDGA KLTGEAGNAYNEVRDYAIKVVSSENKKLSQTAVTMDLAMLQTLKNDAMSKLREAKAKLVPEVKPQENPEHQRPTTPAPDTKPI PQPEGKKPSVPDINQEKEKAKLAVATYMSKILDDIQKHHLQKEKHRQIVALIKELDEFKKQALSEIDNVNTKVEIENTVHKIFADMD AVVTKFKKGLTQDTPKEPDNKKPSAPKPGMQSPQPEGKKPSVPAQPGTEDKKPSAPKPGMQSPQPEGKKPSVPAQPGTEDK KPSAPKPDMPQSPQPEGKKPSVPAQPGTEDKKPSAPKPGMQSPQPEGKKPSVPAQPGTEDKKPSAPKPDMPQSPQPEGKKPS VPAQPGTEDKKPSAPKPDMPQSPQPEGKKPSVPEINQEKEKAKLAVATEKKLPSTGVASNLVLEIIGLLGLIGTSFIAMKRRK----- -
psp C8. 1	MFASKSERKVHYSIRKFSIGVASVAVASLVMGVSVVHATENEGSTQAATSSNMANKSQTEQGEINIERDKAKTAVSEYKEKKVSEIYT KLERDRHKDTVDLVNKLQEIKNYLNKIVESTSTIEIQGLITTSRSLDEAVSKYKKAPSSSSSSGSSTKPETPQPETSKPEVKPEPETPK PEVKPEPETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPELETPKPEVKPELETPKPE VKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVK PEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPDNSKPQADDDKPPSTPNNSKD KQSSNQASTNENKKQGPATNPKKSLPSTGSISNLALAIAGLLTLAGATILAKKRMK----- ----- ----- -----
psp C8. 2	MFASKSERKVHYSIRKFSIGVASVAVASLVMGVSVVHATENEGSTQAATSSNMANKSQTEQGEINIERDKAKTAVSEYKEKKVSEIYT KLERDRHKDTVDLVNKLQEIKNYLNKIVESTSKIEIQGLITTSRSLDEAVSKYKKAPSSSSSSGSSTKPETPQPETSKPEVKPEPETPK PEVKPEPETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPELETPKPEVKPELETPKPE VKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVK PEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPDNSKPQADDDKPPSTPNNSKD KQSSNQASTNENKKQGPATNPKKSLPSTGSISNLALAIAGLLTLAGATILAKKRMK----- ----- ----- -----
psp C8. 3	MFASKSERKVHYSIRKFSIGVASVAVASLVMGVSVVHATENEGSTQAATSSNMANKSQTEQGEINIERDKAKTAVSEYKEKKVSEIYT KLERDRHKDTVDLVNKLQEIKNYLNKIVESTSTIEIQGLITTSRSLDEAVSKYKKAPSSSSSSGSSTKPETPQPETSKPEVKPEPETPKPEV KPEPGTPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPELETPKPEVKPELETPKPEVK EPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVKPEP ETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPDNSKPQADDDKPPSTPNNSKDKQS SNQASTNENKKQGPATNPKKSLPSTGSISNLALAIAGLLTLAGATILAKKRMK----- ----- ----- -----
psp C8. 4	MFASKSERKVHYSIRKFSIGVASVAVASLVMGVSVVHATENEGSTQAATSSNMANKSQTEQGEINIERDKAKTAVSEYKEKKVSEIYT KLERDRHKDTVDLVNKLQEIKNYLNKIVESTSKIEIQGLITTSRSLDEAVSKYKKAPSSSSSSGSSTKPETPQPETPKPEVKPEPETPK PEVKPEPEKPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPELETPKPEVKPELETPKPE VKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVK PEPETPKPEVKPELETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPEP ETPKPEVKPDNSKPQADDDKPPSTPNNSKDKQSSNQASTNENKKQGPATNPKKSLPSTGSISNLALAIAGLLTLAGATILAKKRMK ----- ----- -----
psp C9. 1	MFKSNHERRMRYRIRKFSVGVASVAVASLVMGVSVVHATEKEGSTQAATSFNRNGNSQAEQREELDSERDKARKEVEEYVKKIVGE SYAKSTKKRHTNTVALVNELNNIKNEYLNKIVESTSESQILMMESRSKVDKAVSKFEKDESSSSSSSSSSTKPETPQENPEH QKPTTPAPDTKPSQPEGKKPSVPDIYPGIEKAKLAVATYMSKILDDIQKHRQIVALIKELDELKKQALSEIDNVNTKVEIENTVHKIFA DMDAVVTKFKKGLTQDTPKEPGNKKPSAPKTGMQSPQPEVKPQPKNQNLNRNQKQNLNRNLEKPKPEVKPQPEKPK PEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEK

	PKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPDNSKPQ ADDDKKPSTTNNLSKDKQPSNQASTNEKATNPKKSLPSTGSGISNLALEIAGLLTAGATILAKKRMK----- ----- -----
psp C9. 2	MFKSNHERRMRYRIRKFSVGASVAVASLFMGSVVHATEKEGSTQAATSFNRNGNSQAEQRGELDLERDKAMKAVSEYVGKMMV RDAYVKSDDRHRKNTVALVNQLGNIKNRYLNEIVHSTSKSQLQELMMKSQSEVDEAVSKFEKDSFSSSSSGSSTKPTQPENPEH QKPTTPSPDTKPSQPEGKKPSVPDINQEKEKAKLAVVTYMSKILDDIQNIICRKKKNIVRLLALIKELDELKKQALSEIDNVNTKVEIEN TVHKIFADMDAVVTFFKKGLTQDTPKEPGNKKPSAPKTGMQPSQPEVKPQPKNQNRNLNRNQKNQNRNLNRNLEKPKPEVK PQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQLEKPKPEVKPQPEKPKPEVNPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPE VKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPK PDNSKPQADDDKKPSTTNNLSKDKQPSNQASTNEKATNPKKSLPSTGSGISNLALEIAGLLTAGATILAKKRMK----- ----- -----
psp C9. 3	MFKSNHERRMRYRIRKFSVGASVAVASLFMGSVVHATEKEGSTQAATSFNRNGNSQAEQREELDSERDKARKEVEEYVKIVGE SYAKSTKKRHTNTVALVNELNNIKNEYLNKIVESTSESQQLIMMESRSKVDEAVSKFEKDSFSSSSSGSSTKPTQPENPEHQKPT TPAPDTKPSQPEGKKPSVPDIYPGIEKAKLAVATYMSKILDDIQKHRQIVALIKELDELKKQALSEIDNVNTKVEIENTVHKIFADMD AVVTFFKKGLTQDTPKEPGNKKPSAPKTGMQPSQPEVKPQPKNQNRNLNRNQKNQNRNLNRNLEKPKPEVKPQPEKPKPEVK PQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVNPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPE VKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPDNSKPQADDDK KPSTTNNLSKDKQPSNQASTNEKATNPKKSLPSTGSGISNLALEIAGLLTAGATILAKKRMK----- ----- -----
psp C9. 4	MFKSNHERRMRYRIRKFSVGASVAVASLFMGSVVHATEKEGSTQAATSFNRNGNSQAEQRGELDLERDKAMKAVSEYVGKMMV RDAYVKSDDRHRKNTVALVNQLGNIKNRYLNEIVHSTSKSQLQELMMKSQSEVDEAVSKFEKDSFSSSSSGSSTKPTQPENPEH QKPTTPSPDTKPSQPEGKKPSVPDINQEKEKAKLAVVTYMSKILDDIQNIICRKKKNIVRLLALIKELDELKKQALSEIDNVNTKVEIEN TVHKIFADMDAVVTFFKKGLTQDTPKEPGNKKPSAPKTGMQPSQPEVKPQPEKPKPEVKPQLEKPKPEVKPQPEKPKPEVNPQ PEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPDNSKPQADDDKKPSTTNNLSKDKQPSNQASTNEKATN PKKSLPSTGSGISNLALEIAGLLTAGATILAKKRMK----- ----- -----
psp C1 0.1	MFKSNHERRMRYRIRKFSVGASVAVASLFMGSVVHATEREGSTQAATSSKMANESQIERRKAAEQAIVSLSGYMTSLINDGLDT SDSRFEELLKRAKGIVEEYRKIDGASDREKIEKLQEGQTKLDELVDKFKKGLSSSEQNGHATPKDPSRNDGVQGGDDSSVGQGEQ PGASNQKNPESVPPTASGETDSPTKPKTSNDSVNSLERELKEVKETAKTTLNTYMLTRLKQENPGVFWFADLLRESKESVEKYKRM CDEASSKENVENLVKEAQEIEALVVKHKGGRGIDLERTKAKTVIAKYLTGLDDIKKLNKKEQHINTVELIKKLGDIKRTYLYKDESTQ KPTTGTGPESHQTRFSNLKWLSSSSVRILINQKSAPEKEHQNPPTAPDTKPSQPEGKKPSVPDINQEKEKAKLAVATYMSKIS DDIQKHHLQKEKHRQIVALIKELDELKKQALSEIDNVNTKVEIENTVHKIFADMDAVVTFFKKGLTQDTPKEPGNKKPSAPKPGMQ PSPQPEVKPQLEKPKPEVKPQPEKPKPEVKPQLEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQ PEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQPEKPKPEVKPQLEKPKPEVKPQLEKPKPEVKPQLEKPKPDNSK PQADDDKKPSTTNNLSKDKQPSNQASTNEKATNPKKSLPSTGSGISNLALEIAGLLTAGATILAKKRMK----- -----
psp C1 1.1	MFASKNERKVHYSIRKFSIGVASVAVASLFMGSVVHATEKEVTTQVATSSNKANKSQTEHMKAAQVDEYIEKMLSEIQLDRRKH TQNVGLLTKLGAIKTEYLRGLSVSKEKSTAELPSEIKEKLTAAFEQFKKDTLKSQKKAQAEQKAKDQKEAQEIEALVKKHGREIDL RKKAKAAVTEHLKLLNDIEKNLKEQHTHTVELIKNLKIDIEKTYLHKLDESTQKAQLQKLAIESQSKLDEAFSKFKNLSSSSNSGSS KPTQPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPELETPKPEVKPEPETPKPE VKPELETPKPEVKPELETPKPEVKPELETPKPEVKPELETPKPEVKPELETPKPEVKPELETPKPEVKPELETPKPEVKPELETPKPEVK EPETPKPEVKPEPETPKPEVKPELETPKPEVKPELETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPEIPKPEVKPDNS KPQADDDKKPSTPNNLSKDKQSSNQASTNENKKQGPATNPKKSLPSTGSGISNLALEIAGLLTAGATILAKKRMK-----

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ps pC 11. 2	MFASKNERKVHYSIRKFSIGVASVAVASLFMGSVVHATEKEVTTQVATSSNKANKSQTEHMKAAKQVDEYIEKMLS EIQLDRRKHTQNVGLLTKLGAIKTEYLRGLSVSKEKSTAELPSEIKEKLTAAFEQFKKDTLKSGKKVAEAQKKAKDQKEA KQEIEALIVKHKGREIDLDRKKAKAAVTEHLKLLNDIEKNLKKEQHTHTVELIKNLKDIEKTYLHKLDESTQKAQLQKLI AESQSKLDEAFSKFKNGLSSSSNSGSSTKPETPQPETPKPEVKPELETPKPELETPKPEVKPELETPKPEVKPEPETPKPE VKPELETPKPEVKPEPETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPELETPKPELETPKPEVK PELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPELETPKPEVKPELETPKPEVKPEPETPKPE VKPEPETPKPELETPKPEVKPELEIPKPEVKPDNSKPQADDDKKPSTPNNLSKDKQSSNQASTNENKKQGPATNKPCKS LPSTGSISNLALAIAGLLTAGATILAKKRMK----- -----
ps pC 11. 3	MFASKNERKVHYSIRKFSIGVASVAVASLFMGSVVHATEKEVTTQVATSSNKANKSQTEHMKAAKQVDEYIEKMLS EIQLDRRKHTQNVGLLTKLGAIKTEYLRGLSVSKEKSTAELPSEIKEKLTAAFEQFKKDTLKSGKKVAEAQKKAKDQKEA KQEIEALIVKHKGREIDLDRKKAKAAVTEHLKLLNDIEKNLKKEQHTHTVELIKNLKDIEKTYLHKLDESTQKAQLQKLI AESQSKLDEAFSKFKNGLSSSSNSGSSTKPETPQPETPKPEVKPELETPKPEVKPELETPKPEVKPELETPKPEVKPELETP PKPEVKPELETPKPEVKPELETPKPEVKPELETPKPEVKPDNSKPQADDDKKPSTPNNLSKDKQSSNQASTNENKKQGP ATNKPCKSLPSTGSISNLALAIAGLLTAGATILAKKRMK----- ----- -----
ps pC 11. 4	MFASKNERKVHYSIRKFSIGVASVAVASLFMGSVVHATEKEVTTQVATSSNKANKSQTEHMKAAKQVDEYIEKMLS EIQLDRRKHTQNVGLLTKLGAIKTEYLRGLSVSKEKSTAELPSEIKEKLTAAFEQFKKDTLKSGKKVAEAQKKAKDQKEA KQEIEALIVKHKGREIDLDRKKAKAAVTEHLKLLNDIEKNLKKEQHTHTVELIKNLKDIEKTYLHKLDESTQKAQLQKLI AESQSKLDEAFSKFKNGLSSSSNSGSSTKPETPQPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVKPELETPKPE PKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPELETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPE VKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPKPEVKPEPETPKPEVKPE LETPKPEVKPELETPKPEVKPEPETPKPEVKPELETPKPEVKPELEIPKPEVKPDNSKPQADDDKKPSTPNNLSKDKQSSN QASTNENKKQGPATNKPCKSLPSTGSISNLALAIAGLLTAGATILAKKRMK----- ----- -----

Supplementary Table 5.1. PspC variants (PspC1.1-PspC11.1)

Chapter 6. Discussion and future work

6.1 Summary of investigation

This project aimed to determine whether streptococcal pathogens target inhibitory LILR for immune evasion. Streptococcal pathogens such as *S. agalactiae*, *S. pyogenes*, and *S. pneumoniae* are particularly adept at evading the human immune system and causing invasive diseases. Virulence factors produced by these opportunistic pathogens inhibit key components of innate immune system such as neutrophils, macrophages, and complement. Identifying these immune evasion molecules and characterising their mechanism of action is not only important for understanding streptococcal pathogenesis but also to provide knowledge of which molecules to target in future therapeutic strategies. Whilst some mechanisms of immune evasion are well characterised, a relatively more recent and less well understood strategy is the targeting of inhibitory immune receptors on human immune cells. LILR are important immunomodulatory immune receptors that regulate innate immune responses (Lewis Marffy and McCarthy 2020; Abdallah et al. 2021). The inhibitory members are known to be targeted by several pathogenic bacteria (Abdallah et al. 2021); however, the bacterial ligands involved are unknown. Here, the interactions of *S. agalactiae*, *S. pyogenes*, and *S. pneumoniae* with inhibitory LILR were investigated and the bacterial ligands involved in terms of binding and function were characterised. Furthermore, the role FH plays in *S. agalactiae* immune evasion was explored.

A screen of pathogens against LILR by the McCarthy lab had identified potential interactions of *S. pyogenes* with LILRB2 and *S. agalactiae* with LILRB3; however, the bacterial ligands involved were unknown. In Chapter 2, the M3 protein of *S. pyogenes* was identified as a ligand for LILRB2. M proteins are perhaps the best characterised *S. pyogenes* immune evasion molecules that promote virulence through several mechanisms. The characterised mechanisms include inhibition of antibody opsonisation, suppression of complement activation, and the suppression/lysing of phagocytes (Carlsson et al. 2003; Horstmann et al. 1988; Valderrama and Nizet 2018). Thus, the work outlined in Chapter 2 further characterises the host-pathogen interactions of M proteins, and identifies an additional potential immune evasion mechanism i.e. via suppressing immune cell activation through engagement of inhibitory receptors. As no study has yet identified a bacterial ligand for the LILRB2 receptor, this work provides an opportunity to fully characterise how the targeting of LILRB2 by bacteria impacts immune responses and virulence during infection.

Initially, recombinant (r)LILRB2 was demonstrated to bind to *emm3*-type *S. pyogenes* strains, in which rLILRB2 bound to 9/10 *emm3* types screened. Having established that the rLILRB2 ligand(s) was likely a surface protein

and was blocked by an alternative M3 ligand (collagen I and IV), next it was shown that M3 protein on the bacterial surface was necessary for LILRB2 interaction utilising a *L. lactis* model. This was followed by assays demonstrating that rM3 protein directly bound to rLILRB2. rM3 protein was produced using an *L. lactis* expression system and M3-LILRB2 interaction was tested utilising a Dynabead biochemical assay. Having established LILRB2 directly bound M3, the functional consequences of the interaction were investigated. The interaction of M3-expressing *S. pyogenes* and *L. lactis* with LILRB2 did not enhance bacterial resistance to whole blood or neutrophil killing. Further studies, utilising neutrophil and/or macrophage immune cells in other physiological environments are required to fully understand the significance of LILRB2-M3 interactions for immune evasion. However, rLILRB2 did reduce M3-mediated adherence to collagen I and collagen IV. Thus, secreted LILRB2 may reduce *S. pyogenes* adherence and prevent colonisation, a crucial first step in pathogenesis.

Another potential interaction the McCarthy group discovered was *S. agalactiae* with LILRB3. Whilst LILRB3 is ubiquitously expressed by myeloid cells, only one ligand has recently been identified - APOE4 (Zhou et al. 2023). This contrasts with LILRB2 and LILRB1, which have several known ligands (Abdallah et al. 2021). Several proteomic and mAb studies indicate that LILRB3 is differentially regulated during immune cell activation (Lewis Marffy and McCarthy 2020). Furthermore, bacterial pathogens *E. coli* and *S. aureus* have been shown to interact with LILRB3; however, the bacterial ligands of LILRB3 from these species are yet to be identified (Nakayama et al. 2007). Thus, determination of bacterial ligands that interact with LILRB3 remains a priority to not only to understand bacterial pathogenesis but also elucidate the function of LILRB3, a key regulator of innate immune responses.

In Chapter 3, β protein of *S. agalactiae* was identified as a LILRB3 ligand. The β protein is expressed on the surface of several virulent serotypes (Lindahl, Ståhlhammar-Carlemalm, and Areschoug 2005), but predominantly in the serotypes Ia and Ib that are associated with invasive disease (Francois Watkins et al. 2019; Slotved and Hoffmann 2020; Hsu et al. 2022; Bobadilla et al. 2021). Previous studies comparing wildtype and $\Delta\beta$ protein strains demonstrated the importance of β protein expression for virulence by enhancing resistance to neutrophil phagocytosis and killing (Carlin, Chang, et al. 2009). This was proposed to function through interaction of β protein with the inhibitory Siglec-5 receptor (Carlin, Chang, et al. 2009). However, subsequent investigations have revealed that β protein binds multiple inhibitory receptors (CEACAM1 (van Sorge et al. 2021) and LILRB3 (this study)) and regulatory complement factors (Lindahl et al. 1990) independently. This raises new questions on interpretation of previous data, and it makes determining the interactions responsible for immune evasion of β protein difficult to ascertain.

To overcome this, the approach taken was to determine the LILRB3 binding interface on β protein and utilise this knowledge to produce bacterial strains that expressed β protein with the LILRB3 interaction abrogated, whilst retaining interaction with other binding partners, such as Siglec5 and CEACAM1. Initially, we identified two LILRB3 binding domains – B6C and B75KN – expressed by β protein. By producing and screening a panel of β protein mutants for binding to recombinant LILRB3, two β protein residues – E357 in B6C domain and L218 in B75KN domain – were identified that appeared to be important for binding of β protein to LILRB3. However, a *L. lactis* strain transformed to express β protein with B6C^{E357A} and B75KN^{L218A} mutations still bound rLILRB3. This

suggests additional β residues are involved in the LILRB3 binding, and/or other domains/structures in full length β protein are involved in maintaining the conformational shape of the LILRB3 binding domain. As a result of this setback, the functional consequences of β -LILRB3 interaction for neutrophil phagocytosis using a rLILRB3 blocking assay was used with preliminary data suggesting that β protein binding to LILRB3 suppresses bacterial phagocytosis by neutrophils.

In Chapter 3 the B75KN domain of β protein was identified as a LILRB3 ligand whose interaction may be involved in immune evasion. Thus, in Chapter 4, in collaboration with the Geisbrecht lab, we characterised the structure of the LILRB3 binding domain – B75KN – and utilised this knowledge to determine its role in immune evasion. The β protein is a multifunctional virulence factor whose crystal structure has not been solved preventing full understanding of how its virulence is mediated. However, the β protein domain Igl3 has been solved, which led to the discovery of a novel Ig-fold (Igl3) that binds CEACAM1 and CEACAM5 (van Sorge et al. 2021). Characterisation of the Igl3 structure enabled discovery of Igl3 homologs in additional bacterial pathogens, which were subsequently shown to interact with CEACAM1, such as R28 expressed by *S. pyogenes* (Catton et al. 2023). Thus, determining the structure of the B75KN domain and its role in immune evasion, was important for characterising β protein virulence. Furthermore, the structural knowledge could be used to identify additional LILRB3 binding bacterial ligands. Interestingly, structural analysis of the B75KN domain revealed a FH binding motif that led us to explore this interaction in the context of immune evasion (Xu et al. 2022).

These data showed that B75KN binds FH and that this interaction inhibits complement activation. Through a series of biochemical assays, the C-terminal domain of B75KN, called B75KN-CTD, was shown to bind FH. The crystal structure of the B75KN-CTD domain was solved, and a FH binding motif – QHLQKKN – was identified. A *L. lactis* strain that expressed β protein with the FH binding motif knocked out was constructed, and this motif was shown to be necessary for FH binding. The functional consequences of this interaction for complement activation were then assessed and the data revealed that β protein, via the B75KN domain, suppressed complement activation by FH-dependent inhibition of C3b deposition and FH-dependent accelerated decay of the C3bBb convertase. To assess whether binding FH and complement inhibition promoted immune evasion, whole blood and neutrophil phagocytosis assays were performed. These experiments demonstrated that B75KN expression was important for β protein mediated inhibition of neutrophil phagocytosis; however, this was independent of FH binding.

Finally, we utilised structural knowledge of the B75KN-CTD from Chapter 4 to identify additional bacterial ligands that may interact with LILRB3. In Chapter 5, a bioinformatical approach identified PspC proteins that may bind LILRB3. PspC are key virulence proteins expressed by *S. pneumoniae* (Kerr et al. 2006), and similar to β protein, they are considered immune evasion molecules whose virulence is proposed to be mediated by inhibiting complement through sequestering FH (Quin et al. 2007). Recombinant forms of *S. pneumoniae* PspC proteins - rPspC3, rPspC4 and rPspC7 – were demonstrated to bind to rLILRB3. Bacterial proteins are often shared between species, particularly *Streptococcus*, and bacterial proteins contain structurally homologous domains that confer binding phenotypes (Lindahl, Ståhlhammar-Carlemalm, and Areschoug 2005; van Sorge et al. 2021). Thus, databases were searched for structurally homologous domains to B75KN-CTD and these searches identified the

surface-bound PspC proteins of *S. pneumoniae* as sharing structural homology. A panel of rPspC proteins were produced and tested for LILRB3 interaction, with rPspC3, rPspC4 and rPspC7 identified as potential LILRB3 ligands. The interaction of LILRB3 with PspC3 was further explored. However, LILRB3 did not bind PspC3.4 when expressed by *S. pneumoniae*. Furthermore, blocking of LILRB3-PspC3.4 interactions did not alter macrophage internalisation of *S. pneumoniae*. However, preliminary data suggested that LILRB3 expression by macrophages is downregulated during *S. pneumoniae* infection. This suggests that LILRB3 is differentially expressed on macrophages surfaces in response to *S. pneumoniae* infection.

6.2 General Summary

In summary, streptococcal pathogens were demonstrated to target inhibitory LILR receptors via expression of surface proteins associated with virulence. This study also identified the first bacterial ligands of inhibitory LILR: *S. pyogenes* binds LILRB2 via M3 protein and *S. agalactiae* binds LILRB3 via β protein (**Fig 6.1**). *S. pneumoniae* may also bind LILRB3 via a subset of PspC. Furthermore, in collaboration with the Geisbrecht lab, it was shown that FH binds β protein via a loop located in the B75KN domain (the same domain that binds LILRB3). Investigation of the functional consequences of bacterial inhibitory LILR interactions revealed some unexpected results. The engagement of LILRB2 by *S. pyogenes* did not enhance survival in whole blood. However, further studies are required to ascertain whether LILRB2-M3 engagement may be important for immune evasion in these or other physiological environments. In contrast, the host may secrete soluble LILRB2 to inhibit *S. pyogenes* colonisation via M3-dependent interactions with collagens. Preliminary data suggest that *S. agalactiae* engagement of LILRB3 via β protein suppresses phagocytosis of bacteria by neutrophils. Interestingly, FH binding to β protein did not enhance immune evasion of bacteria in whole blood. This suggests an alternative role for FH- β protein interaction or functional redundancy in the immune evasion strategies of β protein of *S. agalactiae*. Finally, *S. pneumoniae* PspC proteins directly interacted with LILRB3, which may result in downregulation of LILRB3 on macrophage surfaces.

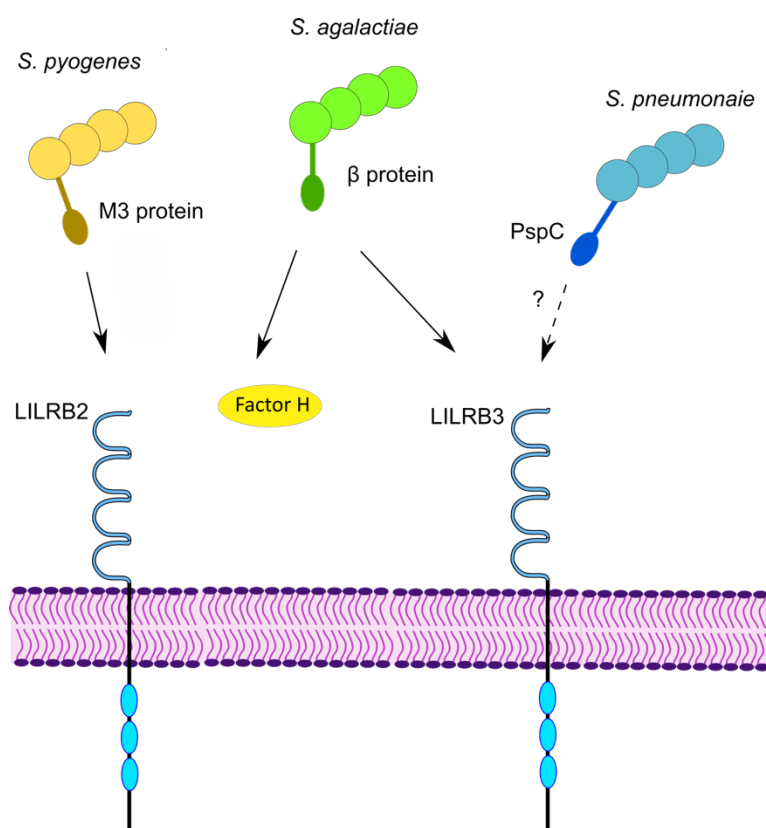


Figure 6.1 Thesis summary diagram. A schematic depicting the key interactions discovered and explored in this thesis. M3 protein expressed by *emm3* *S. pyogenes* directly interacts with LILRB2. In addition, *S. agalactiae* express β protein that directly binds LILRB3 via two distinct domains: B6C and B75KN. Interestingly, the B75KN-CTD region of β protein also binds FH. Finally, exploratory work has potentially identified a subset of PspC proteins expressed by *S. pneumoniae* that interact with LILRB3. Having determined the bacterial ligands that interact with inhibitory LILR, further work is required to fully determine the receptor-ligand binding interfaces and to understand the functional consequences of these interactions.

6.3 Future work

6.3.1 How do bacteria target inhibitory LILRB2 and LILRB3?

It is essential to determine the binding interface of the bacterial ligands and inhibitory LILR to understand whether LILR engagement promotes bacterial immune evasion. The bacterial ligands that interact with LILR, β protein for LILRB3 and M3 protein for LILRB2, are multifunctional, engaging multiple immune evasion molecules (Lindahl, Ståhlhammar-Carlemalm, and Areschoug 2005). Thus, protein knockout assays can not be used to determine the functional consequences of LILR engagement. It is therefore necessary to characterise the binding interface. Identifying the key residues of bacterial ligands required for LILR interaction would enable the

construction of the appropriate tools to specifically interrogate whether bacteria engage inhibitory LILR for immune evasion, similar to the approach taken in Chapter 4 with FH and β protein (Xu et al. 2022).

Having demonstrated that LILRB2 directly binds M3, it would be interesting in future work to define the LILRB2-M3 binding interface. To determine the LILRB2 binding domain expressed by M3, the binding of rLILRB2-His to truncated rM3 proteins should be tested. This would help to localise the binding site of LILRB2 in M3 and define the mechanism of interaction. Subsequently, suitable M3 protein variants could be engineered that do not interact with LILRB2. If M3 protein variants could be expressed in bacterial strains, this could be used as a valuable tool to measure the specific functional impact of the M3-LILRB2 interaction. This would be reflective of the experiments performed on β protein-FH in Chapter 4. Alternatively, *in silico* docking of the N-terminal domain of M3 onto LILRB2 D1-D4 domains could predict the binding mechanism. However, the crystal structure of the M3 N-terminal domain is yet to be solved. Furthermore, the predicted structure for the N-terminal region of M3, available on AlphaFold (Accession: AF-W54835) has low predictive confidence. Therefore, additional structural information is required for M3 to predict the mechanism by which it interacts with LILRB2.

In complementary assays, it would be useful to define the M3 binding site on LILRB2. LILRB2 has 4 Ig domains called D1, D2, D3, and D4 (Shiroishi et al. 2003). Currently, we do not have any knowledge of the LILRB2 domains required for M3 engagement. To localise the LILRB2 binding domains, biochemical experiments are required to test rM3-His binding to truncated versions of rLILRB2-His with specific domains deleted. Of note, LILRB2 recognises a) HLA-A, -B, -C and -G via D1 and D2, b) Angptl3 via D1 and D4 (Yu et al. 2018; 2018), and c) specific RIFIN molecules expressed by *P. falciparum* via D1, D2 and D3 (Sakoguchi et al. 2021). Thus, these ligands, and/or defined anti-LILRB2 monoclonal antibodies, could be used to test for their ability to competitively inhibit LILRB2 interaction with M3. If these ligands do competitively inhibit M3 binding to LILRB2, then this would indicate the LILRB2 domains involved in M3 binding.

When determining the LILRB3- β protein binding interface, biochemical assays using alanine mutants identified β protein residues B6C^{E357} and B75KN^{L218} as potentially important for rLILRB3-His binding to B6C and B75KN domains, respectively. However, B6C^{E357A} and B75KN^{L218A} mutations in the full length β protein, on the surface of *L. lactis*, did not abrogate binding to LILRB3-His. Thus, there are clearly other residues that are important for the LILRB3 and β protein interaction. Indeed, our alanine mutant screen also revealed the involvement of residues D219, and to a lesser extent Y213 and H230, in B75KN. In future work, I would produce *L. lactis* strains that expressed β protein with additional mutations of B75KN^{D219A}, B75KN^{Y213A} and B75KN^{H230A} residues. To fully abrogate LILRB3- β interaction, it would also be important to better define the B6C and LILRB3 interface. In future assays, additional B6C mutant proteins should be produced and screened for LILRB3 binding. Another important consideration to investigate is whether the mutant B6C and B75KN-CTD recombinant proteins produced retain their native conformation. This is necessary to ensure that any abrogation of LILRB3 binding observed is due to disruption of a LILRB3 binding domain, and not due to loss of overall native conformation. The gold standard techniques for assessing protein structures is by x-ray crystallography or nuclear magnetic resonance imaging; however, these approaches are expensive and labour intensive. There are however several approaches that are less labour intensive and could be used to determine whether specific mutations in B6C and B75KN-CTD caused

loss of overall native conformation including circular dichroism, Fourier-transformed infrared spectroscopy, and UV/vis fluorescence microscopy (Manta et al. 2011).

These investigations have focused on the β protein side of the host-pathogen interface. However, identifying the LILRB3 domains targeted by β protein is also important. Recent work by the Shi lab identified APOE4 as a novel endogenous LILRB3 ligand (Zhou et al. 2023). Through expression of truncated LILRB3 proteins that expressed single or combinations of D1, D2, D3, and D4 Ig-like extracellular domains of LILRB3, the Shi lab demonstrated that APOE4 binds to D2 and D4 domains of LILRB3 (Zhou et al. 2023). Ongoing research in the McCarthy lab is taking a similar approach to identify the Ig-like domains of LILRB3 that bind β protein. This analysis has revealed that both the B6C and B75KN domains of β protein bind to D3, D4 and/ or the D3-D4 loop in LILRB3 (unpublished data, Rumpret, Fu & McCarthy). Having identified the Ig-like domains involved in β protein binding, a bioinformatical approach could be used to predict the mechanisms by which B6C and B75KN dock onto LILRB3 domains D3 to D4. This would likely identify the LILRB3 residues involved in binding β protein domains. A panel of LILRB3 mutants using site-directed mutagenesis of the identified LILRB3 residues could be constructed and subsequently be screened for B6C and B75KN binding using the established biochemical approaches. Alternatively, solving the crystal structure of LILRB3 in complex with β protein, or LILRB3-D3-D4 domains with B75KN-CTD/B6C, using x-ray crystallography or cryo-EM, would fully define the LILRB3- β protein binding mechanisms. Ultimately, this knowledge is important to generate β protein and/or LILRB3 variants that fail to form interactions, and subsequently to perform experiments that assess the specific function of β protein-LILRB3 interaction.

6.3.2 Do *S. pyogenes* and *S. agalactiae* interact with inhibitory LILRB2 and LILRB3, respectively, for immune evasion purposes?

Whilst studies have demonstrated that virus and protozoan target inhibitory LILR for immune evasion (Saito et al. 2017; Yu et al. 2018; Cosman et al. 1997), there is currently no study demonstrating that bacteria utilise inhibitory LILR for immune evasion. Thus, determining whether *S. pyogenes* and *S. agalactiae* target inhibitory LILR for immune evasion is a priority. Furthermore, investigating the functional consequences sheds light on precisely how bacterial ligands exert their immune evasion properties.

Building on work from Chapter 2, future investigations should determine the functional consequences of LILRB2-M3 engagement for bacterial immune evasion. Whilst the role of M3 protein for virulence has been demonstrated, the precise mechanism of how M3 protein mediates immune evasion during invasive disease remains unclear. Invasive *emm3* strains of *S. pyogenes* have been shown to inhibit neutrophil recruitment for immune evasion purposes (Stetzner et al. 2015). Furthermore, purified M3 protein may have anti-phagocytic properties (Hong et al. 1995). However, the ligands and immune cell signalling mechanisms involved in M3-

mediated immune evasion are still to be characterised. Thus, determining the functional consequences of LILRB2-M3 presents an opportunity to fully characterise an M3-mediated immune evasion mechanism for a highly virulent *S. pyogenes* clone.

Given that differences in bacterial immune evasion due to blocking the LILRB2-M3 interaction were undetectable in whole blood assays, future work is required to explore the functional consequences using purified immune cell assays. The advantage of such assays is that they could be more sensitive and therefore detect LILRB2-M3 mediated functions. In particular, a comparison of neutrophil killing and effector responses to M3-expressing *L. lactis* or *S. pyogenes* after blocking LILRB2 interaction is required given the immunoregulatory functions of LILRB2 in neutrophils (Baudhuin et al. 2013). Alternatively, and because neutrophils cannot be genetically manipulated, it would be interesting to use neutrophil-like cell lines such as PLB-985 that are transfected to over express LILRB2 (Marin-Esteban et al. 2012; Amulic et al. 2017). A comparison of the capacity of wildtype and LILRB2-expressing PLB-985 to perform effector functions and kill bacteria (such as M3-expressing *L. lactis*) may reveal the role LILRB2-M3 engagement has for immune evasion.

It would also be interesting to explore the functional consequences of LILRB2-M3 interaction for monocytes, and macrophages. Macrophages are important in phagocytosis and killing of *S. pyogenes* during infection. However, *S. pyogenes* can also survive and replicate in macrophages, potentially for immune evasion purposes (Goldmann et al. 2004; Valderrama and Nizet 2018). Thus, it would be interesting to explore whether the M3-LILRB2 interaction was involved in *S. pyogenes* intracellular survival. For example, LILRB2 knockout macrophage cell lines, such as THP-1, could be generated using CRISPR-Cas9, and then used to compare *S. pyogenes* intracellular survival between LILRB2 knockout and wildtype THP-1 cells (Lindner et al. 2021; Gažová et al. 2021). Alternatively, because the promonocytic U937 cell line does not express LILRB2 (unpublished findings in McCarthy lab), the functional impact of this interaction could be assessed by comparing the capacity of M3-expressing *S. pyogenes* or *L. lactis* to survive phagocytosis and/or killing by differentiated LILRB2+ U937 cells and wildtype U937 cells.

Finally, it would be important to explore the role of LILRB2-M3 interaction during pathogenesis using an *in vivo* mouse model. Paired immune receptor B (PIR-B) is the mouse ortholog of LILRB2 receptor (Martin et al. 2002). As Pir-B contains six Ig-like domains whilst LILRB2 expresses four Ig-like domains, it would initially be important to test whether PIR-B interacts with M3. If Pir-B does not interact with LILRB2, a transgenic mouse expressing human LILRB2 would be required to investigate the role of M3-LILRB2 interactions in *S. pyogenes* invasion and disease processes (Torii et al. 2008; Ukai et al. 2017). These transgenic mice have recently been developed (Chen et al. 2018).

In Chapter 3, preliminary functional assays, using competitive rLILRB3 blocking, suggests that β protein engagement with LILRB3 suppresses phagocytosis of bacteria by neutrophils. In future, further rLILRB3 blocking assays should be performed to confirm this preliminary finding and utilise the same rLILRB3 blocking assay to assess other neutrophil effector functions such as NETosis and ROS production. In addition, it would be interesting to assess whether blocking of LILRB3 similarly inhibits killing of β protein-expressing bacteria by

neutrophils and/or in human whole blood. Though this could be achieved using an anti-LILRB3 blocking mAb, this would first require the identification of a mAb that could block β protein binding to LILRB3. Next, it would be important to determine whether the LILRB3- β protein interaction functions synergistically with other host- β protein interactions. β protein engages other inhibitory immune receptors expressed on neutrophils including CEACAM1 and Siglec-5 (Carlin, Chang, et al. 2009; Fong et al. 2018; van Sorge et al. 2021). All or some of these β protein interactions could drive β -dependent immune evasion properties. Thus, to specifically interrogate the functional consequences of LILRB3- β protein interaction, and to understand whether these interactions function synergistically, construction of *L. lactis* strains that expressed mutant β proteins with individual or combined LILRB3, CEACAM1 or Siglec-5 interactions knocked out should be performed. This would then allow comparison of neutrophil functions against a panel of *L. lactis* strains to understand if β protein interactions with inhibitory immune receptors are synergistic.

Due to the broad expression profile of LILRB3 on human immune cells, β protein may suppress effector functions of other myeloid immune cells involved in innate immune responses including basophils, eosinophils, monocytes, macrophages, dendritic cells, and osteoclasts (Pfistershammer et al. 2009; Yu et al. 2018; Huang et al. 2010; Bashirova et al. 2022; Mori et al. 2008). Furthermore, LILRB3 engagement is known to inhibit other myeloid immune cell effector functions. For example, knockdown of LILRB3 expression in monocytes and macrophages was shown to inhibit ROS production and killing of *P. aeruginosa* (Ming et al. 2019). In addition, engagement of LILRB3 has been shown to inhibit basophil secretion of immunomodulatory factors including histamine, cysteinyl leukotrienes and IL-4 generation (Yu et al. 2018) which are important for activation and maturation of macrophages, dendritic cells, and B cells (Min, Brown, and LeGros 2012). Thus, β protein mediated LILRB3 engagement may be advantageous for *S. agalactiae* through suppression of multiple myeloid cell functions beyond neutrophils. It would be interesting, in future work, to assess the functional consequences of β protein engagement of LILRB3 with *ex vivo* monocytes and macrophages, and/or utilise cells lines such as THP-1 or U937 cells.

Determining the precise role of LILRB3 for immune evasion remains a challenge due to LILRB3 being paired with LILRA6. LILRA6 is an activating LILR receptor that associates with ITAM-containing adaptor proteins such as FcR γ upon ligation (Abdallah et al. 2021). It shares significant homology to the extracellular domain of LILRB3, with antibodies often being cross-reactive (Zhao et al. 2020; Hirayasu et al. 2021). Indeed, the genetic similarity between LILRB3 and the activating receptor LILRA6 and their SNPs prohibits the use of SNP-based genome-wide association studies with disease outcomes (Hirayasu et al. 2021). There are currently no studies specifically interrogating LILRA6 function; however, LILRA6 copy number variation has been associated with increased risk to atopic dermatitis, a disease characterised by abnormal or sustained activation of the skin innate inflammatory system (Yu et al. 2018). Thus, LILRA6 likely plays a proinflammatory role, that counterbalances LILRB3-mediated inhibitory functions.

In future assays it would be important to determine whether LILRA6 also binds β protein. To test this, I would produce rLILRA6 and/or construct LILRA6 expressing cell lines and measure their interaction with β protein, rB6C, rB75KN and β -expressing bacteria. Given the sequence similarity of LILRB3 and LILRA6, the hypothesis proposed

would be that interactions are formed between β protein and LILRA6. If LILRA6 does indeed bind β protein, it would be important to determine the impact of this interaction on anti-*S. agalactiae* responses. Importantly, because LILRA6 is not expressed by human neutrophils (Zhao et al. 2020), it would be expected that β protein suppresses neutrophil effector functions as it would interact with an inhibitory receptor (LILRB3) but not the paired activating receptor (LILRA6). In contrast, macrophages express both LILRB3 and LILRA6 (Bashirova et al. 2022). To determine the role of LILRB3/LILRA6 for macrophages, genetically manipulated THP-1 or U937 cell lines to over-express LILRB3, LILRA6, or LILRB3 and LILRA6 could be used in infection assays. This would allow functional assays to be performed to dissect binding effects for each receptor. Interestingly, whilst most antibodies are cross-reactive against LILRB3 and LILRA6, certain antibodies such as MKT5.1 can differentiate between LILRB3 and LILRA6 surface expression (Hirayasu et al. 2021). Thus, I would select LILRB3 and LILRA6 alleles to express that could be distinguished by antibodies, and therefore, enable more accurate assessment of LILRB3/LILRA6 expression.

Work in Chapter 4, demonstrated that FH and LILRB3 bind the same domain. It would be interesting to determine whether these interactions were antagonistic or synergistic. To test this, β protein-expressing *L. lactis* could be coated with FH, and the adherence to LILRB3-expressing U937 or LILRB3-reporter cell lines measured. If FH does indeed block LILRB3 engagement with β protein, it suggests that they are antagonistic, and it would be interesting to explore the functional consequences of β protein-LILRB3 interaction in low FH environments such as mucosal environments. Alternatively, if FH and LILRB3 both bind to β protein simultaneously, it is likely that they work synergistically. To test this, competitive blocking using rLILRB3 could be used to compare phagocytosis of FH coated bacteria expressing wildtype β or $\beta\Delta$ FHBD by neutrophils.

6.3.3 What are the signalling pathways triggered by bacteria binding to inhibitory LILR?

Determining the intracellular signalling pathways involved upon inhibitory receptor engagement is essential for understanding inhibitory receptor function during infection and more broadly for immune homeostasis. For example, Siglec-5's role in suppressing myeloid cell activity was demonstrated by pull down and microscopy assays which showed that ITIM phosphorylation led to recruitment of SHP-1/SHP-2 phosphatases which resulted in inhibition of Fc ϵ RI-mediated calcium flux and serotonin release in myeloid cells (Avril et al. 2005). Elucidation of the intracellular signalling provides the link between ligand-mediated activation and immune cell effector responses. Importantly, identification of the signalling pathways involved in inhibitory receptor engagement may also indicate the transcriptional environment and suggest immune effector functions that are being suppressed. For example, subsequent work on Siglec-5 signalling and function in T cells demonstrated that β protein-mediated engagement of Siglec-5 suppressed activity of transcription factors NFAT and AP-1 (Vuchkovska et al. 2022). In addition, identifying the signalling pathways inhibitory receptors interact with offers

additional potential therapeutic targets that may enhance responses to infection. Thus, in future work investigating the signalling pathways triggered by bacterial engagement of inhibitory LILR would be important to determine how bacterial ligands may suppress immune cell activation and further characterise LILR functions for immune homeostasis.

Identifying M3 as a ligand for LILRB2 enables investigation of LILRB2-mediated immune cell signalling mechanisms involved during *S. pyogenes* infection. Initially, a LILRB2 reporter cell line could be constructed to assess whether M3 protein induces cross-linking of LILRB2 receptors, similar to that described in Chapter 3 for LILRB3. Additionally, immune cells lines described above (PLB-985 or THP-1 or U937) could be incubated with rM3-His and the effects on LILRB2 phosphorylation and signalling could be assessed. For example, it would be important to assess the phosphorylation of ITIM residues in LILRB2 and the recruitment of key signalling molecules such as SHP1/2 by Western blot (Fanger et al. 1998). Finally, it would be important to assess the activation of the proinflammatory signalling pathway STAT1/NF- κ B and secretion of inflammatory cytokines TNF- α and IL-6 which are modulated by LILRB2 signalling (Singh et al. 2022).

Previous work has demonstrated that mAb engagement of LILRB3 suppressed IgA-mediated neutrophil effector functions such as phagocytosis and ROS production (Zhao et al. 2020). However, phosphorylation of LILRB3 ITIM domains and signalling pathways involved remain unclear. Thus, it would also be interesting to utilise the assays described above to determine the intracellular signalling involved in β protein engagement with LILRB3.

6.3.4 Do other bacteria interact with inhibitory LILR?

Initial work suggested that LILRB3 interacts with PspC proteins of *S. pneumoniae*. Discovering additional bacterial ligands is essential for determining whether targeting inhibitory LILR is a general mechanism immune evasion employed by streptococcal pathogens. If these pathogens, as well as *S. aureus* and *E. coli* (Nakayama et al. 2007), utilise common immune evasion mechanisms, it opens the possibility of designing therapeutics that target several pathogens simultaneously. Furthermore, discovering additional ligands for LILRB3 will aid characterisation of the LILRB3 receptor, and offer more insights in how innate immunity is regulated, and/or subverted during infection.

Biochemical screens revealed that rLILRB3-Fc bound to rPspC3.4. However, rLILRB3-His did not bind to the TIGR4 strain - a PspC3.4 expressing strain. These confounding results may be due to surface structures present on the *S. pneumoniae* surface that blocks the interaction or PspC3.4 under the experimental conditions used. An alternative approach would be to probe a TIGR4 lysate, or cell wall extract, for binding to rLILRB3-His or -Fc by Western blot. If rLILRB3-His or -Fc binding was confirmed by Western blot, next it would be important to assess whether low PspC3.4 surface expression of the TIGR4 strain tested explained why rLILRB3-His binding was not observed by flow cytometry. To test PspC3.4 expression anti-PspC3.4 serum or using a monoclonal antibody could be used as previously described (Marshall et al. 2021). Anti-PspC3.4 serum could be produced by

inoculating mice or rabbits with rPspC3.4-His. If PspC3.4 expression is low, alternative TIGR4 variants and/or PspC3.4 expressing *S. pneumoniae* strain would be tested.

To further explore whether surface expressed PspC3 bound LILRB3, it would be important to test rPspC3.4 binding to LILRB3-expressing cell lines, such as wildtype U937 cells or LILRB3-expressing U937 cells. In addition, I would test whether fluorescently labelled wildtype and Δ pspC3.4 TIGR4 *S. pneumoniae* strains adhere to the same pair of U937 cell lines by flow cytometry. As a complementary assay, co-localisation of wildtype TIGR4 with LILRB3 on the LILRB3-expressing U937 cell lines could be measured by fluorescence microscopy. For both the flow cytometry and fluorescence microscopy assays, anti-LILRB3 and/or anti-PspC3.4 mAbs would be useful to block the interaction, thus showing that PspC3.4-LILRB3 adhesion was specific.

The initial screen of a panel of rPspC-His also identified binding of LILRB3 to PspC7 and PspC4. Utilising the assays described above, it would be interesting to explore whether LILRB3 bound to PspC7- and PspC4-expressing *S. pneumoniae* and the functional consequences of the interaction. Well-characterised *S. pneumoniae* strains that express PspC7 (G54) and PspC4 (CT22F 11295) are widely available for such assays. Furthermore, it would be important to screen additional PspC subtypes. PspC1, PspC2, PspC10, and PspC11 could be produced utilising the same approach taken for other PspC subtypes. Previous attempts to amplify these genes from the strains provided by the Rosconi lab and express corresponding proteins in *E. coli* had failed. Therefore, in future several primers could be designed for each gene and/or utilise at least two different strains for each PspC, to ensure increase the chance of amplifying the genes. In addition, it would be interesting to determine whether LILRB3 binding was PspC subtype dependent. To address this, a panel of PspC3, PspC4, or PspC7 variants would be produced and LILRB3 binding measured. An improved understanding of the direct interaction of several PspC's and LILRB3 would be important to understand which domains/structures are necessary for LILRB3 and whether PspC expresses multiple LILRB3 binding domains similar to β protein.

6.3.5 Do bacteria regulate LILRB3 expression?

Initial data suggested that LILRB3 expression on THP-1 macrophages surfaces is downregulated in response to *S. pneumoniae* infection, although further repeats are required. As previously discussed, LILRB3 expression is differentially regulated during infection (Ming et al. 2019). Thus, the downregulation of LILRB3 may represent a host response to *S. pneumoniae* infection. If LILRB3 surface expression is downregulated in response to *S. pneumoniae*, it would be important to next investigate whether PspC3.4 binding to LILRB3 induced downregulation. To test this, a comparison of LILRB3 surface expression after infecting with wildtype or PspC3.4 knockout mutant would be important. This would determine whether LILRB3 regulation is a general host mechanism to *S. pneumoniae* infection or a PspC-mediated mechanism. Having established that *S. pneumoniae* induces LILRB3 downregulation via a PspC-dependent or -independent mechanism, it would be interesting to determine how THP-1 macrophages regulate LILRB3 surface expression. Several inhibitory immune receptors

such as PD-1, PD-L1, and CTLA4 are internalised and recycled or degraded to modulate surface expression levels (He and Xu 2020). Whilst LILRB3 surface expression is regulated in response to the external environment, the mechanism is unknown. To understand whether LILRB3 is recycled in endosomes or degraded in response to *S. pneumoniae*, co-localisation of LILRB3 receptors after infection to endosomes or lysosomes could be assessed by fluorescence microscopy.

6.3.6 What is the role of bacteria sequestering FH?

Several bacterial pathogens and the ligands involved in sequestering FH have been identified (Lambris, Ricklin, and Geisbrecht 2008). The general assumption is that bacteria sequester FH to enhance immune evasion by inhibiting complement. Indeed, increased levels of FH binding to *S. pyogenes* are correlated with enhanced resistance to whole blood killing (Haapasalo et al. 2008). However, this and many other studies investigating FH binding for immune evasion have been confounded by the multifunctionality of FH-binding bacterial proteins. For example, the streptococcal FH binding proteins such as M protein, PspC, and β protein bind several host immune factors simultaneously. Consequently, the studies using gene knockdown measure the impact of each protein in virulence or immune evasion, and do not measure the specific impact of FH-binding to virulence or immune evasion. In the case of β protein, whilst FH is known to bind *S. agalactiae* via the β protein, the precise role of sequestering FH in immune evasion and/or virulence remains unclear. To address this, β -FH binding interface was determined, and this knowledge was utilised to construct the necessary tools to specifically interrogate the role β sequestering of FH has for immune evasion. The binding of FH was localised to a loop in the B75KN domain of β protein, and this interaction resulted in inhibition of complement activation. In particular, it reduced both C3b deposition and accelerated C3 convertase decay. However, β protein sequestering of FH did not enhance bacterial resistance to whole blood killing or neutrophil phagocytosis. Thus, the role of FH during β protein-mediated immune evasion remains unclear.

In future assays, it would be interesting to test different physiological environments, to determine whether FH binding may be more important during different stages of invasive disease. *S. agalactiae* must first colonise mucosal surfaces, prior to invasive infection, which exhibits a different environmental niche than in the blood. Indeed, there is evidence that the environment determines the ability of immune evasion molecules to function. For example, M1 protein mediated non-opsonic binding of IgG does not occur in the IgG rich environments such as blood plasma, but only in low IgG environments such as saliva (Nordenfelt et al. 2012). Thus, it would be interesting to investigate the role of FH binding to β -protein-expressing bacteria in mucosal environments.

In addition, FH may provide a role for immune evasion during bacterial growth in blood. A recent study, investigating the role of PspC and FH binding, demonstrated using fluorescence microscopy that PspC clustered at the division septum, binding FH and inhibiting complement deposition, to enable bacterial growth in blood (Pathak et al. 2018). As *L. lactis* is susceptible to bacterial killing, this would mean construction of *S. agalactiae*

mutant strains that do not express confounding FH-binding proteins (ShtI and ShtII; (Moulin et al. 2019) but that do express β protein or $\beta\Delta\text{FHBD}$. This would then allow assessment of whether β protein clusters during *S. agalactiae* division and binds FH using fluorescence microscopy.

Alternatively, β protein sequestering FH could have a role in macrophage immune evasion. Interestingly, recent work suggests a possible role for β protein in inhibiting macrophages effector functions (Janžič et al. 2023). A comparison of serotypes and macrophage phagocytosis demonstrated that those serotypes that expressed β protein had the lowest rates of phagocytosis, even when compared with highly virulent serotypes III (Janžič et al. 2023). Thus, it would be interesting to explore whether resistance to macrophage phagocytosis is due to FH binding.

Finally, investigations should be performed to determine whether *S. agalactiae* sequestered FH for non-immune evasion purposes. Whilst bacterial binding to FH is predominantly associated with suppressing complement for immune evasion, there is evidence that this mechanism increases colonisation. A study showed that *S. pneumoniae* PspC binding to FH enhances bacterial adhesion to epithelial cells (Agarwal et al. 2010). Utilising complementary PspC antibody and FH ligand blocking adhesion assays, they demonstrated that FH-coated *S. pneumoniae* had enhanced adhesion to epithelial cells. In future assays, it would be interesting to investigate whether *S. agalactiae* and/or β protein-expressing *L. lactis* coated in FH had enhanced adhesion to epithelial cells.

6.3 Conclusion

In conclusion, the work here has identified, for the first time, bacterial ligands that interact with inhibitory LILR: *S. pyogenes* M3 protein binds LILRB2 and *S. agalactiae* β protein binds LILRB3. Furthermore, in collaboration with the Giesbrecht lab, the β protein LILRB3 binding domain (B75KN) was characterised and FH binding was localised to a disorder loop within the B75KN domain. Finally, initial exploratory work suggests that *S. pneumoniae* likely interacts with LILRB3 through expression of a subset of PspC proteins. Initially, functional studies suggest that β protein engages LILRB3, suppressing neutrophil phagocytosis. Whilst M3 engagement of LILRB2 did not promote bacterial survival in whole blood, further work is required utilising different experimental conditions and models, to fully explore this interaction.

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