Isolation and Preliminary Characterization of Anti-Inflammatory Molecules from the Staphylococcal Cell Wall

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Abstract

The innate immune response to pathogens plays important roles to limit early infection and initiate subsequent adaptive immune mechanisms. Innate recognition of microbes are initiated by pattern recognition receptors (PRRs), germline-encoded receptors which are well known to initiate inflammatory processes upon activation. Toll-like receptors (TLRs) are a large class of PRRs, and have recently been described to also be capable of inducing anti-inflammatory responses. TLR2 activation by *Staphylococcus aureus* peptidoglycan preparations has been shown to induce the production of the anti-inflammatory cytokine IL-10 in human peripheral blood mononuclear cells (PBMCs). Previous work in our laboratory has shown that the pro- and anti-inflammatory responses elicited by *S. aureus* community isolates can be uncoupled mechanistically. We hypothesized that these two responses are resultant from two distinct sets of ligands on the *S. aureus* cell wall. *S. aureus* community isolates were enzymatically digested to prepare staphylococcal cell walls. Size-exclusion chromatography and ion-exchange chromatography were used to fractionate cell wall preparations by size and charge, and fractions of interest were identified by assaying pro- and anti-inflammatory cytokine production following *ex vivo* stimulation of human PBMCs. We found that biochemical separation was able to enrich fractions of cell wall components in IL-10-inducing capacity. Through proteomic analyses of these fractions, proteins that were dependent on Sortase A for cell wall anchoring were identified, and Sortase A was found to be indispensable for IL-10 induction. Additionally, fusion proteins linking the extracellular domains of TLR2, 1, 6, and 10 with human IgG1 Fc domains were generated as tools to purify TLR2 ligands from complex ligand mixtures by affinity chromatography. Anti-inflammatory TLR2 ligands, once found, can be used as templates for the development of novel immunomodulatory strategies.

Résumé

La réponse immunitaire innée joue un rôle important pour circonscrire rapidement la progression d'une infection causée par un pathogène et pour enclencher les mécanismes de la réponse immune adaptative. La reconnaissance des microbes par la réponse innée est médiée par les récepteurs de reconnaissance de motifs moléculaires (PRR), des récepteurs codés par les cellules germinales reconnus pour induire de l'inflammation lorsqu'ils sont activés. Les récepteurs de type Toll (TLR) sont une classe de PRR pouvant aussi induire une réponse anti-inflammatoire. Il a été démontré que l'activation de TLR2 par des préparations de peptidoglycane provenant de *Staphylococcus aureus* pouvait induire la production d'une cytokine anti-inflammatoire, l'interleukine-10 (IL-10), par les cellules mononuclées du sang périphérique (PBMC). Les précédents travaux de notre laboratoire ont démontré que les réponses pro- et anti-inflammatoires induites par des souches sauvages de *S. aureus* provenant d'individus porteurs ne sont pas liées par un mécanisme commun. Nous avons donc émis l'hypothèse que ces deux réponses sont induites par deux types de ligands distincts, présents sur la paroi de *S. aureus*. Afin de vérifier cette hypothèse, nous avons procédé à une digestion enzymatique de souches sauvages de *S. aureus* afin d'en extraire les parois cellulaires. Nous avons ensuite fractionné ces extraits en utilisant les méthodes de chromatographie d'exclusion stérique et de chromatographie à échange d'ions pour ensuite stimuler ex vivo des PBMCs humains avec les différentes fractions afin d'identifier celles contenant des molécules capables d'induire la production de cytokines pro- ou anti-inflammatoires. Nous avons ainsi pu identifier des fractions enrichies en molécules induisant la production d'IL-10. Une analyse protéomique subséquente a démontré que ces fractions contenaient des protéines dont l'ancrage à la paroi bactérienne dépend de la protéine Sortase A, une protéine qui s'avère indispensable à l'induction d'IL-10. Afin de purifier par chromatographie d'affinité les ligands de TLR2 à partir d'une mixture de ligands, nous

avons généré des protéines fusionnant le domaine extracellulaire de TLR2, 1, 6 ou 10 avec le domaine Fc de l'IgG1 humaine. Ces nouveaux outils peuvent être utilisés en combinaison avec des méthodes biochimiques afin de séparer et isoler les ligands présents sur la paroi cellulaire de *S. aureus* qui sont responsables de l'induction d'IL-10. Une fois identifiés, ces ligands de TLR2 aux propriétés anti-inflammatoires pourraient être utilisés comme point de départ dans le développement de nouvelles méthodes d'immunomodulation.

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List of Abbreviations

AD	Assay Diluent
ANOVA	Analysis of variance
APC	Antigen-presenting cell
BCR	B-cell receptor
BSA	Bovine serum albumin
CA-MRSA	Community-acquired methicillin-resistant *Staphylococcus aureus*
CFU	Colony-forming units
CHO	Chinese hamster ovary cells
CLR	C-type lectin receptor
CTL	Cytotoxic T lymphocyte
CV	Column volume
DAMP	Damage-associated molecular pattern
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FPLC	Fast protein liquid chromatography
GI	Gastrointestinal
GNBP3	Gram-negative bacteria binding protein 3
HAI	Hospital-acquired infection
IL-10	Interleukin-10
ILC	Innate lymphoid cells
IRAK-1	Interleukin-1 receptor-associated kinase 1

kDa	Kilodalton
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
MHC	Major histocompatibility complex
MRSA	Methicillin-resistant *Staphylococcus aureus*
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nod-like receptor
NTML	Nebraska Transposon Mutant Library
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PES	Polyethersulfone
PGN	Peptidoglycan
PGRP-SA	Peptidoglycan recognition protein SA
PMSF	Phenylmethanesulfonylfluoride
PRR	Pattern-recognition receptor
PVDF	Polyvinylidene fluoride

RLR	RIG-I-like receptor
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TAK1	TGF-β-activated kinase 1
TCR	T-cell receptor
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptor
TNF (TNFα)	Tumor necrosis factor
TSA	Tryptic-soy agar
TSB	Tryptic-soy broth

Chapter 1 – Introduction

Staphylococcus aureus (*S. aureus*) is a gram-positive bacterium that is a causative agent of a wide range of diseases in different sites of the human body [1]. These diseases include skin infections of varying severity, invasive infections of internal tissues, and toxin-mediated inflammatory diseases [1]. As the most commonly isolated specimen in inpatient samples, *S. aureus* is a particularly problematic bacterium for hospital-acquired infections (HAI), especially in the context of increasing antibiotic resistance [2]. In 2007, a report published by the Centers for Disease Control and Prevention identified *S. aureus* as the most significant cause of serious infectious disease as well as infectious disease mortality in the United States [3].

Paradoxically, *S. aureus* is also chronically and asymptomatically carried in the anterior nares of a significant portion of the population. Estimates of the rates of chronic carriage vary between 20-35% [4-7]. Chronic carriers are more likely to be infected by *S. aureus* compared to non-carriers, particularly by the strain they carry, yet have more favorable clinical outcomes in the case of bacteremia [8]. The ability of *S. aureus* to act as both a pathogen and a commensal qualifies it as a pathobiont, defined as a microbe that is generally safe, but can become pathogenic even in immunocompetent hosts [9]. A key research theme in the study of host-pathogen interactions is trying to understand this dual relationship between *S. aureus* and the immune system.

The determinants of *S. aureus* nasal carriage are unclear. Both host and microbial genetic and environmental factors can influence the outcome of their interactions, leading to

clearance, commensalism, or infection (reviewed in [10]). Factors such as serum vitamin D levels [11], nasal microbiota [12, 13], and expression of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are known to influence nasal colonization [14, 15]. Elucidation of the factors involved in these processes can lead to new insights for host-microbial interactions, as well as form the basis for novel therapeutic strategies.

One potential explanation for *S. aureus* pathobiosis is its ability to influence the host microenvironment. The Madrenas laboratory has previously identified a mechanism by which *S. aureus* is able to induce the production of the anti-inflammatory cytokine interleukin 10 (IL-10) in human monocytes through activation of the pattern recognition receptor (PRR) Toll-like receptor 2 (TLR-2) [16, 17]. Through testing a panel of community *S. aureus* isolates, we found that the ability of these isolates to induce IL-10 in human peripheral blood mononuclear cells (PBMC) does not correlate with the ability to induce the pro-inflammatory cytokine tumor necrosis factor (TNF) [18]. This suggests that the activation of distinct innate immune pathways leads to the secretion of pro- and anti-inflammatory cytokines.

Further studies have investigated the mechanistic uncoupling of pro- and anti-inflammatory cytokines induced by *S. aureus*. We found that IL-10 secretion is dependent on the PI3K-Akt-mTOR pathway, whereas TNF secretion requires p38 [18]. Signaling compartmentalization was also shown to be important, since phagocytosis and phagosome maturation is required for secretion of TNF but not IL-10. While this study

showed that the host pathways for eliciting the pro- and anti-inflammatory responses are distinct, the heterogeneity observed within the community isolates further suggests that different signals given by *S. aureus* lead to these distinct responses.

Though TLR-2 is known to signal from the cell surface to induce pro-inflammatory responses, the Madrenas laboratory previously showed that the IL-10 response to *S. aureus* is also dependent on TLR2 activation [16]. The purpose of the research presented in this thesis is to test the hypothesis that distinct sets of ligands embedded within the cell wall of *S. aureus* differentially regulate the inflammatory environment, and to characterize TLR-2 ligands which lead to anti-inflammatory responses. The following is a review of *S. aureus* pathobiosis as well as its interactions with the host immune system.

1.1 Biology of *S. aureus*

1.1.1 *S. aureus*

S. aureus was first identified in 1880 by Sir Alexander Ogston, a surgeon in Aberdeen, Scotland. By culturing pus from a surgical abcess, he found two distinct groups of cocci, one known as streptococcus which grew in chains, and another which grew in grape-like clusters, which he named staphylococcus [19]. The species name *aureus* is derived from its characteristic golden appearance due to the production of the pigment staphyloxanthin, which also acts as a virulence factor by conferring resistance to reactive oxygen species (ROS) [20]. Further microbiological studies have characterized *S. aureus* as a gram-positive, facultative anaerobe that possesses catalase activity. It often also shows beta-hemolysis when grown on blood agar plates and tests positive for coagulase activity [21].

Human beings are the primary reservoir for *S. aureus*, but it has also been found in livestock and companion animals [22, 23]. A phylogenetic study on *S. aureus* isolates suggested that *S. aureus* lineages are host-adapted and zoonotic events happen at relatively low frequencies, and that in fact the reverse, transmission events from humans to animals, happens more frequently [24]. However, pandemic strains have been traced back to livestock origins [25, 26]. The threat posed by livestock reservoirs of *S. aureus* is particularly concerning given that genes conferring methicillin resistance are suspected of originating in animals [27-29].

S. aureus encodes for a vast array of virulence factors that contribute to its ability to cause disease in human hosts [30, 31]. Many *S. aureus* strains encode superantigens, exposure to which can cause toxic shock syndrome (TSS) and gastroenteritis [32]. Superantigens are pyrogenic exotoxins encoded in mobile genetic elements that bind to certain MHC-II molecules and the variable region of certain T cell receptors (TCR) β-chains to cause large-scale polyclonal T cell activation. The magnitude of this response, where around 25% of T cell clones are activated, compared to 0.0001% of T cell clones activated by a conventional peptide-specific activation [33], results in a cytokine storm. While this state of massive inflammation can be directly deleterious to the host by causing shock and organ failure, the processes required to recover from massive T cell activation, namely T cell deletion and anergy, can be exploited by the bacterium as a way to evade immune responsiveness [34]. As a result of this immune interference, women who develop menstrual TSS do not produce antibodies against the TSST-1 toxin [35].

Further, the excessive production of TNF as a result of superantigen signaling on immune cells suppresses the chemotaxis of phagocytes to the site of infection [36].

Superantigens produced by *S. aureus* include staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEH) and TSST-1, the causative agent of menstrual toxic shock syndrome [37]. Phylogenetically, superantigens have been shown to belong to five related families, which can be distinguished by distinct structural elements [33]. Group I (TSST-1) and group II (SEB-like SAgs) lack a zinc-binding domain in SAg families III, IV, and V. Group II and III (SEA-like) SAgs contain a 9-19 amino acid cysteine loop which is required for emetic activity [38], contributing to superantigen-mediated food poisoning [39]. TSST-1 lacks these cysteine loops, but is unique in its ability to cross mucosal barriers [40]. Group IV superantigens are expressed by streptococci, and Group V contain recently described SAgs displaying similarity to SEI.

In addition to superantigens, *S. aureus* strains may also produce a number of toxins (alpha, beta, and delta toxins) which directly targeting immune effectors for cytotoxic effects [41]. Staphylococcal alpha toxin is particularly important in the pathogenesis of *S. aureus* pneumonia, where it has been implicated in damaging the lung epithelium [42]. Alpha toxin has further been shown to induce cell death in T cell lines, primary human PBMCs, and isolated monocytes by activating the intrinsic apoptotic pathway [43]. Delta toxin has been shown to activate mast cell degranulation, resulting in a hyper-IgE response and inflammatory skin pathologies in a mouse model [44]. These observations

mimic key characteristics of atopic dermatitis, a disease in which 93% of patients are colonized on the lesional skin and 79% of patients are colonized in nasal passages [45].

S. aureus is able to secrete a number of enzymatic virulence factors. Most strains produce coagulase, which is able to catalyze the conversion of fibrinogen to fibrin to promote blood clotting. Coagulase can be secreted or bound to the cell wall. The bound form, also known as clumping factors, can form clots around the bacterium, shielding it from opsonization and phagocytosis [46]. Staphylococcal protein A may serve a similar shielding function by binding to the Fc domain of antibodies and coating the bacterium in host antibodies [47].

S. aureus also produces enzymes which can digest proteins, lipids, nucleic acids, and glycans [30]. In particular, the enzyme hyaluronidase is able to digest hyaluronan, a glycosaminoglycan widely expressed in the extracellular matrix [48]. At face value, *S. aureus* is able to use these molecules as a carbon source, but it is speculated that this enzyme contributes to virulence by increasing the permeability of host tissues to allow microbial invasion. Similarly, exfoliatins are proteases produced by *S. aureus* which target the cadherin desmoglein I [49]. The action of these proteases is to degrade desmosomes that are essential for cell adhesion, thereby contributing to the skin-peeling characteristic of staphylococcal scalded skin syndrome [32].

Of greatest concern in the healthcare context, *S. aureus* isolates widely express beta-lactamases [50]. Beta-lactams are a class of commonly used antibiotics sharing the

structural distinction of a four membered cyclic amide ring. Beta-lactamases deactivate antibiotics by opening this ring, thereby conferring resistance to a wide range of antibiotic drugs [51]. Through selection and the pressures of widespread antibiotic use, *S. aureus* have evolved resistance mechanisms against drugs such as methicillin, oxacillin, and vancomycin – previously considered to be drugs of last resort [52].

1.1.2 *S. aureus* pathobiosis

Traditionally, the interactions between microbes and human hosts have been classified as either commensal or parasitic. In ecological terms, commensalism is defined as a relationship where one organism benefits from another without affecting it. The gut microflora has long been thought to be an example of a commensal relationship, though emerging evidence on the functions of the microbiome has led many to suggest that the relationship is more accurately described as a mutualistic one, where both organisms benefit [53]. In contrast, a parasitic relationship is one where an organism benefits at the expense of the other, as is the case with many pathogenic microbes. *S. aureus* does not fit neatly into these classifications, and is an example of an emerging class of microbes known as pathobionts [9].

S. aureus can cause a variety of diseases in human hosts. The most common site of *S. aureus* infection is the skin, where infection can result in a number of different pathologies such as foliculitis, abscesses, carbuncles and impetigo [1]. *S. aureus* is especially common in patients with atopic dermatitis [54]. Far more serious are invasive infections of tissues such as bones (osteomyelitis), joints (septic arthritis), and heart

valves (endocarditis). The ability of *S. aureus* to form biofilms makes these invasive infections difficult to treat since biofilms allow evasion from host clearance mechanisms as well as increase nutrient availability and resistance to antibiotics [55].

Two particularly concerning facets of *S. aureus* infections are the prevalence of hospital-acquired *S. aureus* infections and rise of antibiotic resistance in *S. aureus* strains. *S. aureus* is the most commonly isolated species from in-patient specimens and the second most common in out-patient specimens [2]. Its hardiness in the environments contributes to nosocomial infections, as sanitation protocols are insufficient to decontaminate environmental reservoirs [56]. Moreover, methicillin resistance appears to be more common hospital acquired infections; in one study tracking *S. aureus* infections in a children's hospital over the course of seven years, the rate of methicillin resistance in isolates was found to be 39% [57]. Although methicillin resistance first became problematic in the nosocomial context, community acquired methicillin resistant *S. aureus* (CA-MRSA) is increasingly become a problem, especially since CA-MRSA seems to be more virulent [58, 59].

Paradoxical to the healthcare problem that *S. aureus* presents, it is also persistently and asymptomatically carried in the anterior nares of an estimated 20-30% of the population [4-7]. Carriage status is not entirely innocuous, since carriers are at an increased risk of *S. aureus* bacteremia, especially from their own strain [8, 60]. However, carriers also show significantly lower all-cause mortality and bacteremia-related deaths compared to non-carrier patients with bacteremia [8, 61]. Although only a quarter of the population carry

S. aureus persistently, almost all adults have detectable titers of antibody against staphylococcal superantigens, indicating that exposure is ubiquitous [61, 62]. From these observations, *S. aureus* can be termed as a 'pathobiont', a microbe which is usually a harmless resident but can act as a pathogen in certain circumstances [63]. This designation is distinct from the category of 'opportunistic pathogens' since pathobionts do not require immunosuppression to become pathogenic.

1.2 Immune recognition of pathogens

1.2.1 Brief overview of the immune system

The function of the immune system is to recognize and defend the host against infectious agents. The immune system has two sets of mechanisms that provide resistance to infection: innate immunity and adaptive immunity. The mechanisms of innate immunity are the first line of defense against microbial invasion, and are activated early during the course of infection in order to contain microbial proliferation, initiate the inflammatory response, and shape the subsequent adaptive immune response [64]. Innate immune cells recognize microbes through germline-encoded receptors known as pattern-recognition receptors (PRRs), which lack the antigen-specificity of the adaptive immune response, but nonetheless confer the ability to differentiate between classes of foreign microbes and activate appropriate downstream responses [64]. Activation of innate immune cells often results in inflammation, which recruits other cells to the site of inflammation. This inflammatory response is essential for generating a fast-acting response to infection, but can also be deleterious to the host by causing tissue destruction and rendering barrier surfaces permeable to microbial penetration [64]. Perhaps the most important function of

the innate immune response is engulfing of microbes by phagocytic cells. Phagocytosis decreases microbial load early in the course of infection, but more importantly, phagocytosis by professional antigen-presenting cells (APC) allows for the presentation of specific antigens from the infectious agent to cells of the adaptive immune system [64].

Adaptive immunity is activated later in the course of infection if innate mechanisms are insufficient to clear the infection. APCs migrate from the site of injury or infection to local peripheral lymphoid organs and present pathogen-specific antigens to resident T cells and B cells, which express a highly diverse repertoire of antigen receptors generated through germline gene rearrangement, other genetic mechanisms (N-additions, P-additions, imprecise joining), and for B cells, somatic hypermutation. Once antigen-specific T cells and B cells are activated, they undergo massive proliferation by clonal expansion. T cells differentiate into $CD4^+$ and $CD8^+$ T cells based on their ability to recognize antigens bound to either MHC-II (CD4) or MHC-I (CD8) molecules [64]. Most $CD4^+$ T cells become T helper cells, which perform assistance functions such as secreting cytokines, B cell antibody class switching, and enhancing phagocyte function. $CD8^+$ T cells become cytotoxic T cells (CTLs), which recognize infected or damaged host cells, and trigger apoptosis through the intrinsic or extrinsic pathways [64]. Certain thymus-independent antigens can stimulate B cells through the B cell receptor (BCR) without the need for antigen processing by APCs and activation of T cells through peptide-MHC:TCR interactions. B cells can differentiate into plasma cells, which produce high specific antibodies in large quantities in order to clear the infection. These effector cells

are short-lived, and after the infection is cleared only a small fraction survive to become memory B cells, which can confer long-term immunity to a previously experienced pathogen [64].

The end-points of microbial colonization at different sites of the body has traditionally been defined as either microbial clearance or persistent infection leading to a chronic disease state. However, recent elucidation of the complex and often context-dependent interactions between hosts and microbes have identified immune mechanisms which promote several alternative outcomes including commensalism and disease tolerance, a harm-reduction strategy to limit tissue destruction and other disease associated with microbial colonization without decreasing microbial load [65]. These mechanisms highlight the true spectrum of possible interactions between hosts and microbes: ranging from mutualism, commensalism, disease tolerance, to pathology. Therefore, key questions moving forward in the study of the immune mechanisms of health and disease are:

a) What factors make individuals susceptible or resistant to disease?
b) What are the mechanisms, both on hosts and microbes, which govern the outcome of their interactions?
c) How can these mechanisms be exploited therapeutically in order to shift microbes away from pathogenicity and towards commensalism or mutualism?

Upon recognition of microbial presence, the innate immune response regulates factors such as antigen-presentation, cell surface signaling, and signaling through soluble

molecules. These signals govern the extent and magnitude of subsequent immune responses [64]. Therefore, continued study of the innate immune response is essential to extend the understanding of host factors that affect the differential outcomes of microbial colonization.

1.2.2 The role of innate mechanisms in the immune response

The innate immune system is comprised of tissues, cells, and signaling pathways which function together primarily to prevent infection and maintain immunological homeostasis at sites exposed to the environment, such as epithelial surfaces. Once these barriers are breached, the innate immune system further serves to limit infection, recruit other immune mediators, and prime the adaptive response [64].

Broadly speaking, intact barrier surfaces serve as the first line of defense against microbial infection. Epithelial surfaces, such as the skin, respiratory tract, and gastrointestinal (GI) tract represent major surface areas that are frequently exposed to the outside environment and thus susceptible to infection. Several mechanisms render these surfaces inhospitable for microbial growth and protect them against deleterious microbial colonization. On the skin, sebaceous glands secrete an acid film, mildly lowering the pH of the skin in order to inhibit microbial growth [66]. In the epithelium of the respiratory and GI tracts, mucus is produced to trap microbes, and coordinated movement sweeps these potential pathogens away (cilia and peristalsis respectively). Secretion of saliva and tears, along with enzymes such as phospholipase A, anti-trypsin, and lysozyme contributes to the maintenance of barrier integrity at various sites. Moreover, the

epithelial layers of the skin and gut are continually renewed by the rapid turnover of cells. These mechanisms maintain the integrity of these barrier surfaces, minimizing the exposure of internal tissues to external microbes [64].

Once anatomical barriers have been breached, cell of the innate immune system take over as the primary mediators of innate immunity. Host tissues are constantly under immune surveillance by tissue-resident leukocytes, especially at sites with a high likelihood of microbial exposure. Macrophages are an especially important tissue-resident cell population which are seeded in various tissues during development [67]. Though collectively referred to as macrophages, these cells are a heterogeneous population with tissue-specific functions, reflecting the heterogeneity of the tissues in which they are established [67]. Macrophages are amongst a number of cells that express PRRs, which detect the presence of microbial-derived molecules. After recognizing that microbes have broken through barrier surfaces, macrophages initiate the inflammatory response, recruiting other circulating immune effector cell types.

Recruitment of phagocytic innate immune cells such as neutrophils and monocytes shortly after infection plays a critical role in containing the initial infection. Neutrophils are the most abundant white blood cell type, and the most prominent phagocyte by proportion. In addition to phagocytosis, they also release antimicrobial factors by degranulation and form chromatin networks known as neutrophil extracellular traps to kill bacteria [68]. Circulating monocytes are recruited to the site of infection, where they differentiate into macrophages and dendritic cells depending on the signals they receive

[69]. Both macrophages and DCs are capable of phagocytosis, an active process by which foreign microbes or damaged host cells and cellular debris are engulfed and digested. Phagocytes express receptors which recognize signs of endogenous damage (damage-associated molecular patterns or DAMPs) as well as signs of exogenous microbial invasion (pathogen-associated molecular patterns or PAMPs) [64]. After recognizing the cell or microbe to be engulfed, phagocytes stretch around and engulf the target, swallowing it in a compartment known as the phagosome. Phagocytes employ a variety of mechanisms to kill microbes, once engulfed. As the phagosome matures, it acquires V-ATPases, NOX2, and various degradative enzymes [70, 71]. These function to acidify the phagosome, generate ROS, and directly degrade bacterial components, though some microbes have mechanisms to evade the hostile environment of matured phagosomes. Innate immune defense mechanisms such as phagocytosis, in conjunction with other innate immune functions such as secretion of anti-microbial molecules, initiation of the complement cascade, and formation of neutrophil extracellular traps, are often able to reduce microbial loads and help contain the initial infection.

Phagocytes undergo different fates after performing their phagocytic functions. Neutrophils die after phagocytosis to become the primary cellular component of pus. Other professional APCs, primarily DCs but to a lesser extent macrophages and B cells, not only phagocytose microbes but also process and present peptides as a complex with the major histocompatibility complex (MHC)-II [64]. The presence of these MHC-II-antigen complexes, expression of co-stimulatory molecules such as CD80 and CD86, as

well as secretion of cytokines, form the basis of innate priming of the subsequent adaptive immune response [64].

A class of lymphoid cells that lack antigen specificity has been recently described. Known as innate lymphoid cells (ILCs), they lack B and T cell receptors and thus do not display antigen-specific responses [72]. ILCs are segregated into three distinct groups based on their cytokine secretion and expression of transcription factors which lead to their development and function. In general, ILCs have been shown to be involved in immune homeostasis, particularly at mucosal sites. Their function is implicated in the pathogenesis of several inflammatory and autoimmune diseases [73]. Although the expression of TLRs on ILCs have not been thoroughly studied, human peripheral blood natural killer cells have been found to express TLR2 and TLR9 [74, 75], and human RORγt+ ILCs were found to express TLR2 and respond to stimulation using synthetic lipopeptides [76].

1.2.3 PRRs in innate immunity

Although innate immune effector cells lack the highly specific antigen receptors of lymphocytes, they nonetheless recognize microbial presence through detection of well-conserved PAMPs by PRRs. These evolutionarily ancient components of the immune system are well conserved receptors that recognize not only molecular patterns associated with microbial presence, but also molecular patterns associated with damaged cells (damage-associated molecular patterns or DAMPs) [64]. Examples of PAMPs are lipopolysaccharides (LPS) on the surface of gram-negative cell envelopes, nucleic acids

from bacteria or viruses, peptidoglycan (PGN), lipoteichoic acid (LTA), and muramyl dipeptide (the carbohydrate backbone of peptidoglycan) from gram-positive bacteria. PRRs are located on a variety of compartments, and can be both membrane anchored or soluble either in the cytoplasm, or secreted into the extracellular space (reviewed in [77]). Some families of PRRs, such as toll-like receptors (TLR) and cytoplasmic NOD-like receptors (NLR) recognize structurally diverse ligands, while others families such as C-type lectin receptors (CLR) and RIG-I-like receptors (RLR) specialize in recognizing specific classes of microbial ligands.

Traditionally, the role of PRRs in innate immunity has been constrained to facilitating early recognition of microbial presence, initiation of downstream inflammatory and phagocytic processes, and providing co-stimulation for the activation of T and B lymphocytes [64]. However, emerging studies on the role of PRRs suggests that they may also play important roles in regulating tissue microenvironments in contact with foreign microbial components [78]. Large mucosal surface areas are constantly in contact with the external environment and exposed to foreign microbes. Emerging evidence suggests that PRR signaling on innate immune mediators can act as the frontline to distinguish commensals from pathogens, and maintain the delicate balance between microbial invasion by pathogenic microbes, and deleterious excessive inflammation from harmless commensals [16, 78, 79]. Due to the diversity in the microbial ligand repertoire they recognize as well as the downstream signals that they activate, PRRs are a powerful innate mechanism for regulating the interactions between host and microbe.

1.2.4 Toll-like receptors

TLRs were the first class of PRR to be discovered in mammals. In 1996, the Toll pathway was discovered in *Drosophila melanogaster* which were deficient in the induction of anti-bacterial peptides but were nonetheless able to mount an anti-fungal response through dorsal, a homolog of the mammalian transcription factor NF-κB [80]. The PRRs associated with the Toll pathway, gram-negative bacteria binding protein 3 (GNBP3) and peptidoglycan (PGN) recognition protein SA (PGRP-SA), were identified in 2000 [81, 82]. A human homolog for Toll, then termed hToll (now known as TLR4), was found to activate NF-κB, and mice deficient in hToll were unable to mount pro-inflammatory cytokine responses to bacterial lipopolysaccharide (LPS) [83, 84]. Since then, the TLR family has expanded to include 13 members, 10 of which are expressed in humans [77].

TLRs are type I transmembrane receptors with distinctive structural elements. The N-terminal extracellular domains of TLR family members contain leucine-rich repeats (LRRs) consisting of α-helices and β-strands linked by loops, which gives them their characteristic horseshoe shape [85]. The C-terminal intracellular domains contain Toll/Interleukin-1 receptor (TIR) domains, which TLRs also share with the adaptor protein myeloid differentiation factor 88 (MyD88) [86, 87].

TLRs can be separated into two categories based on their cellular compartmentalization. TLRs 1, 2, 4, 5, 6, and 10 are located on the plasma membrane with their ectodomains protruding out into the extracellular space, while TLRs 3, 7, 8, and 9 are expressed on

intracellular membranes such as endosomes, phagosomes, and the endoplasmic reticulum, with their ectodomains oriented into the lumen [77]. TLRs are constitutively expressed on antigen-presenting cells of the innate immune system such as monocytes, macrophages, and DCs, as well as selectively expressed on other cell types such as B cells, neutrophils, mast cells, basophils, eosinophils, and epithelial cells [77].

Members of the TLR family can recognize structurally different biological molecules, including nucleic acids, proteins, lipids, lipoproteins, and polysaccharides from a variety of different microbes (Table 1.1) [8]. Some TLRs, such as the ones expressed on intracellular compartments, specialize in a single class of molecules, in this case nucleic acids. Others can recognize a variety of molecules. For example, though TLR4 is widely recognized as the primary sensor of extracellular gram-negative bacteria, it has also been shown to recognize heat-shock proteins [88], fibronectin [89], and even small molecules such as plant diterpenes and the chemotherapy drug paclitaxel [90, 91]. TLR2 displays a similar level of structural diversity in its recognized ligands, ranging from LTA, PGN, lipopeptides and fungal cell wall components (zymosan). Some bacterial components that have been observed to activate TLR2, such as PGN, are complex mixtures of molecules in which the precise TLR2 activator is unknown [92].

Table 1.1: Detection of microbial PAMPs by TLRs and other PRRs of innate immunity. Reproduced from [93].

Species	PAMPs	TLR Usage	PRRs Involved in Recognition
Bacteria, mycobacteria	LPS	TLR4	
	lipoproteins, LTA, PGN, lipoarabinomannan	TLR2/1, TLR2/6	NOD1, NOD2, NALP3, NALP1
	flagellin	TLR5	IPAF, NAIP5
	DNA	TLR9	AIM2
	RNA	TLR7	NALP3
Viruses	DNA	TLR9	AIM2, DAI, IFI16
	RNA	TLR3, TLR7, TLR8	RIG-I, MDA5, NALP3
	structural protein	TLR2, TLR4	
Fungus	zymosan, β-glucan	TLR2, TLR6	Dectin-1, NALP3
	Mannan	TLR2, TLR4	
	DNA	TLR9	
	RNA	TLR7	
Parasites	tGPI-mutin (*Trypanosoma*)	TLR2	
	glycoinositolphospholipids (*Trypanosoma*)	TLR4	
	DNA	TLR9	
	hemozoin (*Plasmodium*)	TLR9	NALP3
	profilin-like molecule (*Toxoplasma gondii*)	TLR11	

TLRs bind to their cognate ligands as dimers, sometimes aided by additional soluble binding factors. Most TLRs, including TLR3, 4, 5, 7, 8, and 9 recognize ligands and signal as homodimers (eg. Two TLR4 molecules form a homodimer to bind LPS). However, TLR2 is unique in that it forms heterodimers with other TLRs to recognize different ligands and activate different downstream effectors. TLRs 1 and 6 are known to heterodimerize with TLR2, and are thought to be in loosely associated complexes with TLR2 on the cell surface until ligand binding triggers stronger dimerization and subsequent signaling. In studies using synthetic lipoproteins, TLR2/1 and TLR2/6 were found to be activated by triacylated and diacylated lipoproteins respectively [77]. The role of TLR10 has not been as strongly studied compared to the other TLRs, since it is a pseudogene in mice due to a retroviral insertion. However, due to its high sequence homology with TLRs 1 and 6, as well as the fact that it lies in tandem with them on the chromosome [94], TLR10 has long been speculated to be a third potential heterodimer

partner for TLR2. Indeed, emerging studies have elucidated its heterodimerization with TLR2, as well as potential roles for TLR10 in the suppression of inflammatory responses [79]. These different heterodimerization partners for TLR2 suggest that TLR2-based receptor complexes may recognize a diverse set of ligands and activate diverse downstream responses.

TLRs play important roles in human disease. When TLR4 was found to be implicated in activating pro-inflammatory cytokine responses to LPS, TLR4 antagonism was investigated as a potential strategy to treat sepsis. Eritoran (E5564), a nonreactive analog of Lipid A, was shown to be safe for use in human patients [95], but was found to be ineffective in patients with severe sepsis [96, 97]. Based on the observation that $TLR4^{-/-}$ mice were protected from H1N1 influenza A-induced lethality [98], Eritoran was also tested in mice models of influenza-induced lethality, where it was found to increase survival as well as decrease lung pathology, viral titres, and pro-inflammatory cytokines [99]. Recognition of endogenous nucleic acids is involved in the pathogenesis of systemic lupus erythematosus [100], and inhibitors of TLRs 7 and 9 have been shown to enhance glucocorticoid-mediated reduction of type I interferons *in vivo* in lupus-prone mice [101]. Inhibition of TLR2 activation using an antibody has been shown to reduce ischemia/reperfusion injury in pig cardiac tissue and following renal transplants in mice [102, 103]. Polymorphisms in TLR genes have been investigated in the susceptibility to certain diseases, but the magnitude of the effects, though statistically significant, tend to be small [104]. Children who are deficient in MyD88 or IRAK4 have increased susceptibility to pyogenic infections [105, 106]. Remarkably, MyD88-deficient children

who survive to adulthood under antibiotic therapy appear to overcome the susceptibility to these infections [105]. Since MyD88 is critical to the signaling pathways of all TLRs except for TLR3, this may cast doubt on the importance of TLRs for resistance to infection. However, there is crosstalk and redundancy in innate detection of microbes by PRRs [93], which coupled with compensatory adaptive immune mechanisms, may explain the protection against infections observed in adults deficient in MyD88.

TLR signaling pathways have also been investigated as potential targets for adjuvants. Early work showed that a lipid A-based adjuvant formulation was able to decrease the inflammation in murine models of leishmaniasis and reduce lethality in mice infected with influenza [107]. A later study showed that TLR4 signaling through LPS enhances B cell activation and function [108]. Furthermore, therapies that were already being used for vaccination and treatment, such as the bacillus Calmette-Guérin tuberculosis vaccine and imiquimod, used to treat genital warts caused by the human papilloma virus, were found to act through TLR pathways [109, 110]. The various roles that TLRs play in the pathogenesis of certain diseases, while being protective in other diseases, highlights the important functions that TLR-mediated responses play in maintaining host immune homeostasis.

1.2.5 TLR-2 signaling

TLR2 signaling is initiated by ligand binding, resulting in stronger heterodimerization. The TIR domains on the cytoplasmic tails of the two-receptor units are brought to close proximity, forming a platform for the further binding of MyD88 and TIRAP/MAL,

downstream TIR-domain-containing adaptor molecules [86, 87, 111]. These TIR domains on the cytoplasmic tail of TLR2 are phosphorylated on tyrosine residues. This has been shown to be essential to signaling, since deficiencies in phosphorylation are correlated with deficient TLR6 heterodimerization as well as MyD88 and TIRAP recruitment [112]. TRAF6 is recruited to the receptor complex, and becomes activated by IRAK-1 which binds to the TRAF domain of TRAF6 [113, 114]. The TRAF6/IRAK-1 complex dissociates from the TLR/MyD88/TIRAP receptor complex and associates with TGF-β-activated kinase 1 (TAK1) and the TAK1 binding proteins TAB1 and TAB2. IRAK-1 is degraded, while the TRAF6/TAK1/TAB1/TAB2 complex moves into the cytoplasm, where it forms a large complex, eventually leading to the activation of TAK1 and NF-κB [115]. NF-κB activation leads to the production of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF [77].

1.2.6 TLR-2 signaling plasticity

The general mechanism of signaling through TLR2 has been known for some time. However, the relative importance of the different possible ligands for TLR2, any differences in signaling mechanism they might induce, and differences in downstream effects remain largely unexplored. Heterodimerization with TLR6 or TLR1 has long been suggested to expand the ligand repertoire without any functional differences in signaling [116], yet some studies suggest that differential signal occurs downstream of these heterodimers [117]. TLR2 is the primary extracellular sensor of gram-positive bacteria in the same way that TLR4 is the primary extracellular sensor of gram-negative bacteria. However, unlike TLR4, for which LPS is recognized as the most important

physiological ligand for detection of gram-negative bacterial presence [77], TLR2 does not have a single ligand which is known to be predominantly important in the context of microbial recognition during infection.

Previously, PGN was thought to be a TLR2 ligand, but highly purified PGN does not elicit NF-κB activity through TLR2 [92], and previous studies that made this claim are regarded as being likely due to other embedded molecules or intracellular NOD2 signaling through recognition of muramyl dipeptide [92, 118]. LTA, a structural glycopolymer which is embedded in the cell membrane but protrudes out through the cell wall, is a likely candidate for TLR2-mediated sensing of bacteria. Though LTA is able to induce cytokine secretion *in vitro*, it is suggested that the concentrations required to do so are not representative of a physiological host-microbe interaction [118]. The same study found that only lipoproteins are sensed by TLR2 at picomolar concentrations, implying that TLR2 activation observed from other cell envelope components (PGN, LTA) are likely due to contamination by lipoproteins. The synthetic lipoproteins PAM2CSK4 and PAM3CSK4 have been extensively used to model TLR2 activation. The crystal structure of TLR2 heterodimers crystallized with these synthetic lipoproteins suggests that the structural basis of binding is insertion of the long acyl chains of the lipoprotein into hydrophobic pockets of TLR2 [119]. Puzzlingly, the acyl chains of lipoproteins are embedded within the bacterial membrane and below the thick peptidoglycan layer [120], calling into question the accessibility of these ligands to TLR2. Moreover, human PBMCs stimulated with these synthetic lipoproteins either separately or in combination are unable to recapitulate the complex cytokine response observed with physiological

TLR2 stimulation [17]. The Madrenas laboratory previously found that when human PBMCs were stimulated with physiological TLR2 ligands such as crude PGN preparations for 18 hours, cytokines detected in the culture supernatant included IL-1β, IL-6, IL12p40, TNF, and IL-10. However, when PBMCs were stimulated with either PAM2CSK4 or PAM3CSK4, only the cytokine IL-6 was observed [17]. This suggests that these synthetic lipopeptides are not necessarily representative of physiological microbial exposure.

TLRs elicit anti-inflammatory responses in addition to the conventional inflammatory responses [16, 18]. TLR activation is well known to induce the production of pro-inflammatory cytokines such as IL-1β, IL-6, IFNγ, and TNF. However, recently it has been recognized that TLR activation can also induce the secretion of anti-inflammatory cytokines such as IL-10 [16-18]. While this has been long observed for TLR4 in response to LPS stimulation, this has been thought of as a homeostatic mechanism to keep inflammation in check [121]. However, in the context of TLR2 activation, we have observed that the anti-inflammatory response varies based on the ligand used for stimulation, and even between different isolates of *S. aureus* [18]. Previous work in our laboratory have shown that the pro- and anti-inflammatory responses can be uncoupled mechanistically. We observed that the induction of pro- and anti-inflammatory cytokines following *S. aureus* stimulation differ in their requirement for the PI3K/AKT/mTOR pathway, cell internalization, and phagosome maturation [18]. This implies that the anti-inflammatory TLR2 response is not a ligand-independent mechanism that indiscriminately suppresses inflammation to limit excessive tissue damage, but a specific

response to a specific set of microbial ligands. Though various microbial preparations have been observed to elicit a TLR2-dependent anti-inflammatory response, there is little known about the precise ligand(s) on gram-positive cell walls which induce this response or the specific heterodimerization state of the receptor complex through which it signals.

Chapter 2 – Rationale and Hypothesis

2.1 Rationale

Although a number of TLR2 ligands have been identified [64], the differences between them in terms of TLR2 signaling, of receptor complexes required for recognition, and of downstream effects in the context of infection and health are unclear. Many embedded components of the gram-positive cell wall, including PGN, LTA, WTA, and lipoproteins, have been suggested as TLR2 ligands. The question of purity and contamination by unintended PAMPs, however, continue to obscure the interpretation of these studies. The use of synthetic lipopeptides for the study of TLR2 activation is one attractive solution taken to circumvent the problem of contamination, but these cannot recapitulate the full range of cytokines elicited after stimulation of TLR2 with physiologically relevant ligands [17].

Also problematic is the use of surrogate measures of TLR2 activation. In many studies, activation of NF-κB is used as the endpoint reading of TLR2 activation [122, 123]. However, it is clear that there are significant differences between the cytokine responses between different TLR2 stimulants [17]. The use of simplified systems was convenient for initial discovery of TLR2 ligands and signaling mechanisms, but belies the diverse array of signals possible upon TLR2 activation.

This is particularly true in light of the recently elucidated anti-inflammatory cytokine induction in response to TLR2 stimulation [16]. We and others have found that these

anti-inflammatory responses are not simply homeostatic in nature, but are likely the result of distinct ligands signaling through TLR2 to activate distinct anti-inflammatory pathways [16, 124]. Key questions that arise from an overview of the body of literature regarding TLR2 function are:

a) What is the molecular nature of these anti-inflammatory TLR2 ligands?
b) What do these specific anti-inflammatory TLR2 ligands reveal about the plasticity of TLR2-dependent responses?
c) How can these anti-inflammatory molecules be exploited therapeutically?

The first step in beginning to address these questions is to isolate bacterial molecules that induce these anti-inflammatory responses. Proximally, the discovery of these anti-inflammatory ligands and pathways can inform the investigation of the full spectrum of TLR2 signaling, especially in light of the emerging evidence that TLRs also recognize a plethora of endogenous molecules. Looking ahead, with autoimmune diseases being on the rise [125, 126], molecules able to specifically induce an anti-inflammatory response can act as templates for novel immunomodulatory therapeutic strategies.

2.2 Hypothesis

Our preliminary results indicate that different TLR2 ligands have differential ability to induce pro- and anti-inflammatory cytokine production. Further, we observed that community isolates of *S. aureus* vary in their ability to induce IL-10, which is not correlated to TNF production. This observation suggests that these two responses can be uncoupled from the level of PAMP expression on *S. aureus* cell envelopes, TLR2 recognition, to host effector pathways. Therefore, we hypothesize that ***the pro- and anti-***

inflammatory responses activated through TLR2 are initiated by two specific sets of ligands on the staphylococcal cell wall.

2.3 Specific Aims

In my M.Sc. project I aimed to:

1) Identify novel anti-inflammatory TLR2 ligands in the cell wall of community *S. aureus* isolates using biochemical and proteomic approaches

2) Develop TLR-Fc fusion proteins to study physiological TLR2 ligands

3 Chapter 3 – Materials and Methods

3.1 *Staphylococcus aureus strains and handling*

Single colonies of *S. aureus* community isolates were picked and used to inoculate 200mL of tryptic-soy broth (TSB). The culture was incubated at 37°C overnight shaking at 200rpm. Bacteria were recovered by centrifugation at 2000 x g for 20 minutes. Bacteria were washed three times with phosphate buffered saline (PBS). To make short-term stocks, bacteria were resuspended in 20mL 25% glycerol in PBS, and frozen at -80 ºC for future use. Colonies were positively identified as *S. aureus* by polymerase chain reaction (PCR) of the *nuc* gene which encodes for a thermonuclease uniquely expressed by *S. aureus*, yielding a 270 bp band [127]. Sortase A deficient strains were obtained from Dr. William Navarro at the University of Toronto.

3.2 *Colony-forming units (CFU) determination of S. aureus*

Short-term stocks of *S. aureus* were thawed and washed three times and subsequently resuspended in PBS. Bacteria were serially diluted seven times through 10-fold dilutions, and 100 µL of the final dilution (10^{-7}) was plated onto tryptic-soy agar (TSA) medium. Plates were incubated at 37°C overnight and counted the next morning. Plates were considered eligible for CFU determination with CFU counts of between 30 and 300 colonies [128]. Two plates were used for each bacteria sample, and the counts were averaged. This average was multiplied by 10^8 to obtain the final bacteria density in CFU/mL.

3.3 *Isolation of S. aureus cell walls by enzymatic digestion*

S. aureus cell walls were prepared as previously described [129]. Washed short-term stocks of *S. aureus* were resuspended in TES buffer (10mM Tris, 1mM EDTA, 25% sucrose, pH=8) at a concentration of 10^9 CFU/mL with lysozyme (1mg/mL) and mutanolysin (0.1mg/mL). The bacteria suspension was incubated at 37°C for four hours with shaking at 200rpm. The bacteria digest was centrifuged at 2500 x g for 15 minutes. The resultant supernatant was designated the cell wall preparation. The pellet was resuspended in TE (10mM Tris, 1mM EDTA, pH=8) + 2% sodium dodecyl sulfate (SDS) and designated the protoplasm preparation (modified from [129]).

3.4 *Fractionation of cell wall preparations by molecular weight*

Cell wall preparations were sequentially filtered using Amicon Ultra Centrifugal Filter Units in order to generate five fractions of varying molecular weight ranges. The whole cell wall preparation was applied to a 100kDa membrane and centrifuged for 25 minutes at 4000 x g. The fraction of preparation retained and concentrated by the filter was designated as the >100kDa fraction, while the fraction which flowed through the membrane was designated as the <100kDa fraction. The <100kDa fraction was then applied to the 50kDa membrane. Using 100kDa, 50kDa, 30kDa, and 10kDa membrane filters, five fractions were generated: <10kDa, 10-30kDa, 30-50kDa, 50-100kDa, and >100kDa.

3.5 *Quantification of protein content by bicinchoninic acid assay*

Proteins were quantified using the BCA Protein Assay Microplate Kit (Thermo Pierce) according to given protocols. Bovine serum albumin (BSA) standards were diluted to 2 mg/mL, 1.5 mg/mL, 1 mg/mL, 0.75 mg/mL, 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL concentrations. 9 µL of standards and diluted samples were aliquoted into a microplate in triplicate. The plate was sealed and pre-incubated for 15 minutes at 37°C. Two hundred sixty µL of the BCA working reagent was dispensed in each well, and the plate was sealed and incubated for 30 minutes. The plate was taken out to cool at room temperature for 5 minutes and read at 570 nm on a microplate reader (Bio-rad 680).

3.6 *Proteinase K treatment of S. aureus cell walls*

Cell wall preparations were treated with 100 µg/mL Proteinase K for four hours at 56°C. Protein digestion was inhibited by adding phenylmethanesulfonylfluoride (PMSF) to a final concentration of 5mM. As an untreated control, cell wall preparations were also treated with Proteinase K and PMSF concurrently, as well as with PMSF alone.

3.7 *Isolation of human peripheral blood mononuclear cells (PBMCs)*

One hundred twenty mL of whole blood was taken from healthy volunteers in accordance with Ethics protocols at McGill and approved by the Faculty of Medicine Research Ethics and Compliance Office. PBMCs were isolated by density gradient centrifugation using Ficoll-Hypaque as described previously [130]. 30 mL of whole blood was layered on top of 20 mL of Ficoll-Hypaque and centrifuged at 740 x g for 30 minutes at room temperature with brakes off. The PBMC layer, found below the plasma and above the Ficoll-Hypaque, was removed, pooled, and washed with PBS. The PBMCs in PBS were

centrifuged at 514 x g for 10 minutes at 4°C to pellet the cells. In order to lyse red blood cells contaminating the PBMC layer, the washed cell pellet was resuspended in 20 mL of hypotonic 0.2% NaCl (w/v) and incubated for one minute. The cell suspension was brought back up to an isotonic salt concentration by addition of 20 mL of hypertonic 1.6% NaCl (w/v). The cell suspension was pelleted by centrifugation at 276 x g for 10 minutes at 4°C. The cells were resuspended in 50 mL PBS and centrifuged at 185 x g for 10 minutes at 4°C. Finally, PBMCs were resuspended in R-10 medium (RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptavidin, 2 mM L-glutamine, and 20 mM HEPES). An aliquot was taken and diluted 1:1 with Trypan Blue to differentiate live cells. The cell suspension aliquot was applied to a haemocytometer to count cell density.

3.8 *Determination of cytokine production by enzyme-linked immunosorbent assay (ELISA)*

Human PBMC were resuspended at a density of 2 x 10^6/mL. One hundred μL of cells at this density was seeded in each well of a 96 well round-bottom cell culture plate to achieve a final count of 2x10^5 PBMC/well. Stimulants were diluted in R-10 medium to a final volume of 100 μL or less. The volume of each well was adjusted up to 200 μL for a final cell density of 1 x 10^6/mL. The cells were incubated for 18 hours at 37°C with 5% CO_2. Cell culture supernatants were collected in clean flat-bottom 96 well plates, and stored at -20°C until analysis by ELISA.

For analysis of cytokine accumulation in cell culture, ELISA kits (Ready-SET-Go! eBioscience) were used. ELISA-ready adsorbent plates (Thermo Scientific) were incubated with capture antibody diluted with 1X coating buffer overnight at 4°C according to manufacturer protocols. Plates were washed 3 times with wash buffer (1X PBS + 0.05% Tween20), dried by blotting on paper, and blocked with 1X assay diluent (AD) for 1 hour at room temperature. The plates were washed 3 times after blocking. Culture supernatants were diluted by appropriate factors to fit the standard curve range using AD and added to the plates. Two-fold serial dilutions of the standards were diluted according to manufacturer certificates of analyses in AD and added to the plates. The plates were incubated with samples and standards for 2 hours at room temperature or overnight at 4°C. After incubation, the plates were washed 4 times. The detection antibody was diluted in AD according to manufacturer protocols and added to the plates. After incubation with the detection antibody for 1 hour at room temperature, the plates were washed 4 times. Streptavidin-HRP was diluted in AD and added to the plates, incubating for 30 minutes at room temperature. Finally, plates were washed 5 times with wash buffer, and color was developed using the supplied TMB substrate solution, stopping the reaction with 2N H_2SO_4 when color is saturated. The plates were read on a Bio-Rad 680 microplate reader at 450 nm using 570 nm as a reference wavelength.

3.9 *Biochemical separation of cell wall preparations using fast protein liquid chromatography (FPLC)*

FPLC-based fractionation of cell wall preparations was done in collaboration with the laboratory of Dr. Albert Berghuis in the Department of Biochemistry at McGill

University. The ÄKTA platform was used for all FPLC separations (General Electric Healthcare).

3.9.1 *Separation of cell wall preparations using size-exclusion chromatography*

Ten mL of the cell wall digest was loaded onto HiLoad 26/60 Superdex 200 pg size-exclusion chromatography columns (GE Healthcare). Degassed PBS was used to elute fractions of the cell wall digest. The column volume (CV) is 320 mL, and 1.2 CV were collected in 14 mL fractions using the Frac-950 automated fraction collector (GE Healthcare)

3.9.2 *Separation of cell wall preparations using anion-exchange chromatography*

Lipids were removed from cell wall preparations by incubation with Cleanascite Lipid Removal Reagent (Biotech Support Group). The Cleanascite-lipid complexes were removed by centrifugation at 1000 x g for 15 minutes at room temperature. Delipidated cell wall digests in TES buffer were loaded onto fractions packed with Q-Sepharose resin (GE Healthcare) containing quaternary ammonium anion-exchange groups. An aliquot of the starting digest, as well as of the flow-through of the column were retained for further analysis. After the initial sample ran through the column, fractions were eluted using increasing salt concentrations. Traces for UV absorption at 220 nm, 280 nm, salt concentration, and conductivity of the solution were recorded. The CV is 220 mL, and 2 CV were collected in 14 mL fractions using Frac-950. The fractions were sterile filtered using Millex-GP syringe filters with 0.22 µm, polyethersulfone (PES) membranes (Millipore). The fractions were returned to isotonic salt conditions by dialysis using Mini

Dialysis Kits with 8 kDa cut-off membranes (GE Healthcare). Fractions were dialyzed against PBS for 4 hours, followed by a change of fresh PBS and a subsequent overnight dialysis.

Fractions of interest were identified and further anion-exchange was done in order to increase the resolution of separation. Fractions of interest were pooled, desalted, and loaded onto fractions packed with Mono-Q resin (GE Healthcare) containing quaternary ammonium anion-exchange groups. An aliquot of the pooled fractions, as well as of the flow-through of the column were retained for further analysis. After the initial sample ran through the column, fractions were eluted using increasing salt concentrations. Traces for UV absorption at 220 nm, 280 nm, salt concentration, and conductivity of the solution were recorded. The CV is 20mL mL, and 2 CV were collected in 1 mL fractions using Frac-950.

3.10 *Construction of TLR-Fc fusion proteins*

Complementary DNA plasmids for TLRs 1, 2, 6, and 10 were a gift from Dr. Lynn Hajjar, University of Washington Department of Comparative Medicine. DNA sequences encoding the extracellular domain of TLRs 1, 2, 6, and 10 were amplified from these plasmids by PCR, using primers which annealed to the DNA sequences after the region coding for the signaling peptide and before the region coding for the transmembrane domain.

TLR2 forward: 5’-GGAATCCTCCAATCAGGCTTCT-3’

TLR2 reverse: 5’-CCTGTCCTGTGACATTCCGACA

TLR1 forward: 5’-GAGTGAATTTTTAGTTGATAGGTCA-3’

TLR1 reverse: 5’-CCAGTTATGTTGCAGGATAATTCAGAC-3’

TLR6 forward: 5’-GAATGAATTTGCAGTAGACAAGTCA-3’

TLR6 reverse: 5’-CCCAGAGTTATGTTGCAGGATAATTC-3’

TLR10 forward: 5’-ATGAGACTCATCAGAAACATTTAC-3’

TLR10 reverse: 5’-TGTGTTGCAAGATAATTCGTGGAG-3’

The DNA sequences encoding the TLR extracellular domains were phosphorylated using T4 polynucleotide kinase (NEB) and purified by gel extraction (Qiagen). For mammalian expression of hTLR-Fc fusion proteins, the pFuse-hIgG1-Fc2 plasmid was used (Invivogen). The pFuse plasmids were transformed into competent *E. coli* (NEB 5-alpha) and plasmids were harvested by miniprep (Qiagen). The pFuse plasmid preparations were digested using the restriction enzyme EcoRV (Fermentas) and dephosphorylated using calf intestinal alkaline phosphatase (NEB). Blunt end ligation was done with a 1:5 molar ratio of plasmid backbone to DNA insert, using T4 ligase (Fermentas). Ligation products were transformed into chemically competent *E. coli* by heat shock and cells were plated on TSA containing 50 µg/mL Zeocin (Invitrogen). Colonies were tested and confirmed for the correct insert in the correct orientation using PCR and Sanger sequencing

(Genome Quebec). Plasmids for transfection were harvested using GeneJET Endo-Free Plasmid maxiprep kit (Thermo Scientific).

3.11 *Transfection of TLR-Fc fusion plasmids*

Chinese hamster cells (CHO) were a gift from Dr. John Orlowski (Department of Physiology, McGill University). CHO cells were thawed and grown on DMEM supplemented with 10% Fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptavidin, and 2 mM L-glutamine. The Lonza Nucleofection electroporation technology was used for transfecting TLR-Fc constructs into Chinese hamster ovary (CHO) cell lines. Electroporation has long been used to transfect DNA into mammalian cells, but high efficiency is difficult to achieve due to the unclear effects of pulse duration and voltage on transfection efficiency [131]. Nucleofection combines cell line-specific optimized electroporation protocols with cell line-specific buffers to yield high transfection efficiencies. The Lonza optimized protocol for CHO cells using nucleofection solution V was used for all transfections. 2 x 10^5 CHO cells were seeded on 6-well plates 2 days before transfection. On the day of transfection, cells were lifted from the plates by treatment with 0.25% Trypsin-EDTA (Life Technologies). Cell were counted, centrifuged, and resuspended at 1 x 10^7/mL in supplemented Nucleofection buffer. 100 µL of the cell suspension and 5 µg of TLR-Fc constructs or 2 µg of control pmaxGFP vector were added to the provided cuvettes. Cuvettes were electroporated in the Nucleofection machine using recommended program (V-23). Transfected cells were immediately transferred to a 6-well plate containing pre-warmed growth medium. Transfection efficiency of the control GFP plasmid was assessed 24 hours after

transfection by flow cytometry. Transfected cells were stained for viability using the Zombie Aqua viability dye (BioLegend). Cell were analyzed on a LSRFortessa flow cytometer. Selection with 1 mg/mL Zeocin was initiated 48 hours post-transfection and continued for two weeks to select for stably transfected clones. Transfectants were stored in batch format for further analysis.

3.12 *Western Blotting*

Culture supernatants were concentrated using a 30 kDa cut-off centrifuge filter (Millipore), mixed with SDS sample buffer, and loaded onto 8% polyacrylamide gels. Samples were run at 80 volts until the dye front reached the separating gel, and 160 volts for the rest of the gel. Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 1X blocking reagent (Roche) diluted in TBS (50 mM Tris, 150 mM NaCl) for 1 hour at room temperature. After blocking, the membrane was blotted with α-TLR2 (clone EPNCIR133, Abcam) at 1:5000 dilution in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween20) supplemented with 5% BSA and 0.02% Sodium Azide. The primary antibody was incubated overnight at 4°C. The membrane was washed 3 times with TBST. The secondary HRP-conjugated α-Rabbit antibody was diluted 1:10000 in TBST supplemented with 5% BSA and incubated with the membrane for 1 hour. The membrane was washed three times following secondary antibody incubation. The chemiluminescence reagent (Roche) was applied to the membrane before imaging using a gel imager (Alpha Innotech Fluorchem).

3.13 *Purification of TLR-Fc fusion proteins*

3.13.1 *Purification of denatured TLR2-Fc*

Two hundred µL of concentrated cell culture supernatants were mixed with 50 µL of Protein A Dynabeads (Life Technologies). The bead mixture was incubated for 10 minutes at room temperature with rotation. The bead-protein complexes were isolated using magnets and washed three times with PBS. Finally, the TLR2-Fc proteins were eluted from the beads by boiling the mixture in 1X SDS sample buffer for 10 minutes.

3.13.2 *Purification of native state TLR2-Fc*

One milliliter of concentrated cell culture supernatants was diluted 1:1 in Protein A binding buffer and applied to pre-packed Protein A-agarose gravity columns (Thermo Scientific). The run-through was allowed to drain through and was collected. The column was washed with 10mL binding buffer. Proteins were eluted with supplied Protein A elution buffer (amine-based, pH=2.8), and 5 x 1 mL fractions were collected for analysis.

3.14 *Statistics*

Statistical analyses of differences between groups was performed by analysis of variance (ANOVA) followed by intergroup comparison with Student's t-test using the Graphpad Prism software. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

4 Chapter 4 – Results

4.1 *Specific Aim 1: To identify novel anti-inflammatory TLR2 ligands in the cell wall of community S. aureus isolates using biochemical and proteomic approaches*

4.1.1 *Staphylococcus aureus cell wall preparations elicit an anti-inflammatory cytokine response*

Community isolates of *S. aureus* were obtained from outpatients from an ear, nose, and throat specialist clinic. Earlier work in the laboratory found that these isolates differed in their ability to induce IL-10, independent of their ability to induce TNF [18]. Amongst these, the S8 isolate was consistently able to induce high levels of IL-10, while the S3 isolate consistently induced low levels of IL-10 [18]. Therefore, these two isolates were chosen to undergo cell wall isolation by enzymatic digestion to determine whether their cell walls can account for the differences in IL-10 induction.

The cell wall and protoplast fractions of S3 and S8 were isolated and quantified using the BCA protein assay. Primary human PBMCs were treated with the fractions for 18 hours, and supernatants were obtained for cytokine quantification by ELISA (Figure 4.1). We found that both IL-10 and TNF were induced by cell wall treatment, whereas only low levels of both cytokines were observed with protoplast treatment. Additionally, the differences between S3 and S8 observed when PBMCs were treated with the whole heat-killed bacteria were recapitulated in the cell wall preparations, as the S8 cell wall was able to induce greater levels of IL-10 at lower concentrations compared to S3.

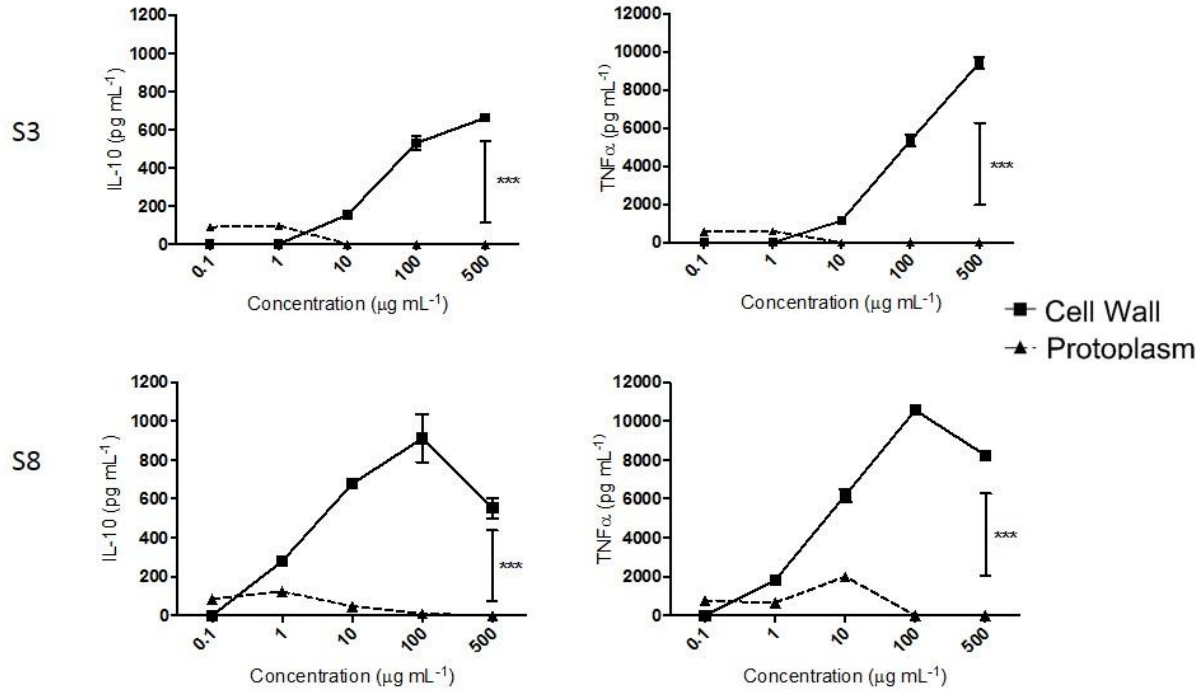

Figure 4.1: Cytokine production by human PBMCs stimulated with cell wall and protoplast preparations from *S. aureus* community isolates S3 and S8. Human PBMCs were seeded in 96 well plates and stimulated with varying concentrations of the cell wall and protoplast fractions. After 18 hours of stimulation, supernatants were collected and analyzed for accumulation of IL-10 (left column graphs) and TNF (right column graphs) by ELISA. Representative experiment shown from three independent experiments. *** indicates $p < 0.001$

4.1.2 *The anti-inflammatory properties of S. aureus cell wall preparations are sensitive to protease treatment*

Although structural studies on TLR2 binding suggest that the basis of ligand-receptor interactions is a hydrophobic interaction between long acyl chains and the hydrophobic pocket in the receptor [119], TLR2 is nonetheless apparently able to bind a variety of different biological molecules [77]. TLR2 is known to recognize a wide variety of biomolecules including lipoproteins, lipoteichoic acid, lipoarabinomannan, and zymosan [77] (which is not expected to contain any long acyl chains). Therefore, cell wall preparations were subjected to protease treatment to examine whether the IL-10-inducing capacity of *S. aureus* cell walls required the presence of intact protein molecules (Figure 4.2).

Proteinase K is a broad spectrum serine protease which targets hydrophobic amino acid residues for cleavage [132]. Cell wall preparations from the S8 isolate were treated with proteinase K for four hours at 56°C and the reaction was inhibited with 5mM PMSF. The cell wall preparation treated with the inhibitor only was tested to ensure that the inhibitor had no direct effect on the IL-10 inducing capacity of the cell wall. As a final control, a zero hour treatment was also assayed, where the cell wall preparation was treated simultaneously with proteinase K and inhibitor before incubation.

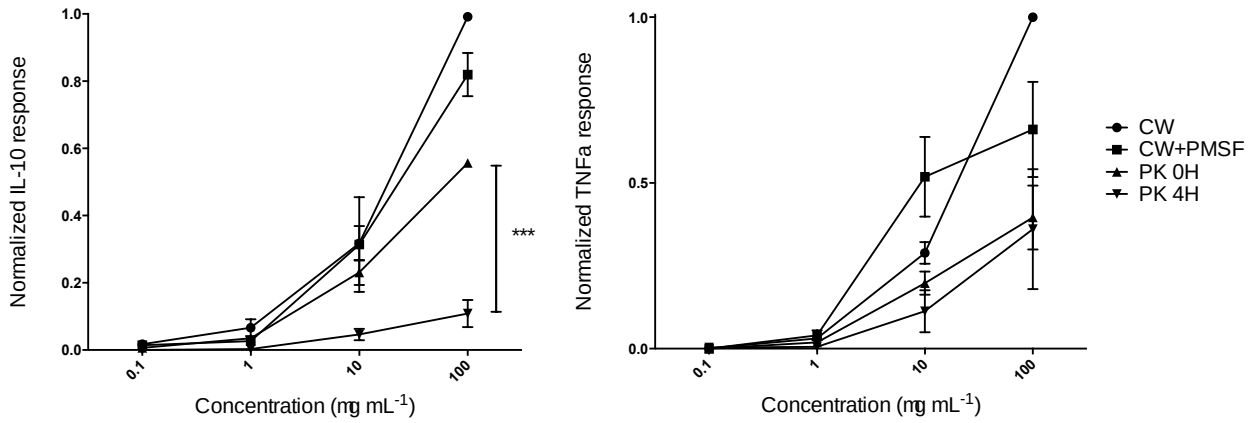

Figure 4.2: Cytokine responses in human PBMCs stimulated with S8 cell wall preparations in the presence or absence of proteinase K treatment. CW: cell wall untreated. CW+PMSF: cell wall with 5 mM PMSF. PK 0H: cell wall treated with 100 µg/mL proteinase K and PMSF inhibitor simultaneously. PK 4H: cell wall treated with 100 µg/mL proteinase K, incubated for 4 hours, then inhibited using PMSF. Data shown are from four donors and were normalized to account for individual differences in cytokine production with error bars showing the standard error of the mean. *** indicates $p < 0.001$

The untreated cell wall and cell wall treated with PMSF alone induced similar levels of IL-10, indicating that PMSF has no direct effects on IL-10 induction. The zero hour treatment condition, intended to assess how well the proteolysis is inhibited by PMSF, showed a slight decrease in IL-10 levels. This indicates that proteinase K inhibition by PMSF is likely leaky, which is consistent with published observations about the instability of PMSF in aqueous solutions [133]. In contrast, the 4-hour treatment condition showed almost complete ablation of IL-10 induction. This indicates that IL-10 inducing ligands in the cell wall preparation were sensitive to proteinase K treatment. The TNF response was observed to vary greatly between donors, with no overall effect seen between the zero hour and 4-hour proteinase K treatment conditions.

4.1.3 *The staphylococcal cell wall components which induce IL-10 include proteins greater than 30kDa in size*

Following the observation that the cell wall-induced anti-inflammatory response was sensitive to proteinase K treatment, we proceeded with determining the size of the IL-10-inducing molecule(s) embedded in the cell wall. In order to do this, molecular weight cutoff centrifuge filters were used sequentially to generate five size range fractions. These fractions were quantified for protein content by BCA assay, and used to treat primary human PBMCs to analyze cytokine production.

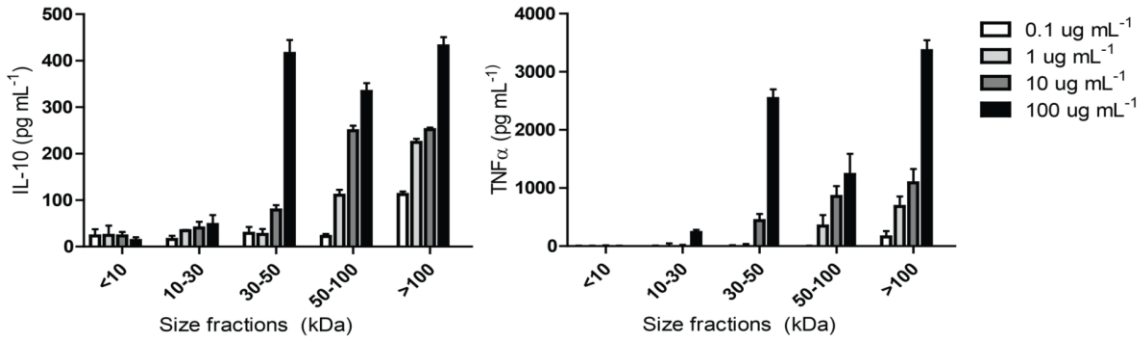

Figure 4.3: Cytokine responses in human PBMCs treated with cell wall preparations fractionated by molecular weight. Staphylococcal cell wall preparations were sequentially filtered using different size cut-offs to generate five fractions of varying molecular weight ranges. These fractions were used to stimulate human PBMCs for measurement of cytokine accumulation by ELISA as before.

Strong IL-10 responses were observed for fractions greater than 30kDa in size (Figure 4.3). The 30-50 kDa fraction induced IL-10 production at the highest concentration but was not dose dependent, where at both the 50-100 kDa and >100 kDa fractions produced the expected dose-response effect. The ability of the 30-50 kDa and >100 kDa fractions to induce TNF mirrored the IL-10 response, whereas the 50-100 kDa fraction was slightly lower. This suggests that the 50-100 kDa fraction may preferentially induce IL-10, since the TNF response for this fraction was relatively lower.

4.1.4 *Size-exclusion chromatography is able to generate fractions which are enriched in IL-10-inducing activity*

The strong IL-10 response observed with the 50-100 kDa fraction of the cell wall, in conjunction with the weak TNF response, suggested that it was possible to separate pro- and anti-inflammatory ligands by molecular weight. Fast protein liquid chromatography was chosen as a higher resolution method to fractionate the cell wall by descending molecular weight. *S. aureus* cell wall preparations were separated using a HiLoad 26/60 Superdex 200 pg column to generate fractions. These fractions were diluted 1:10 and used to treat human PBMCs for cytokine responses.

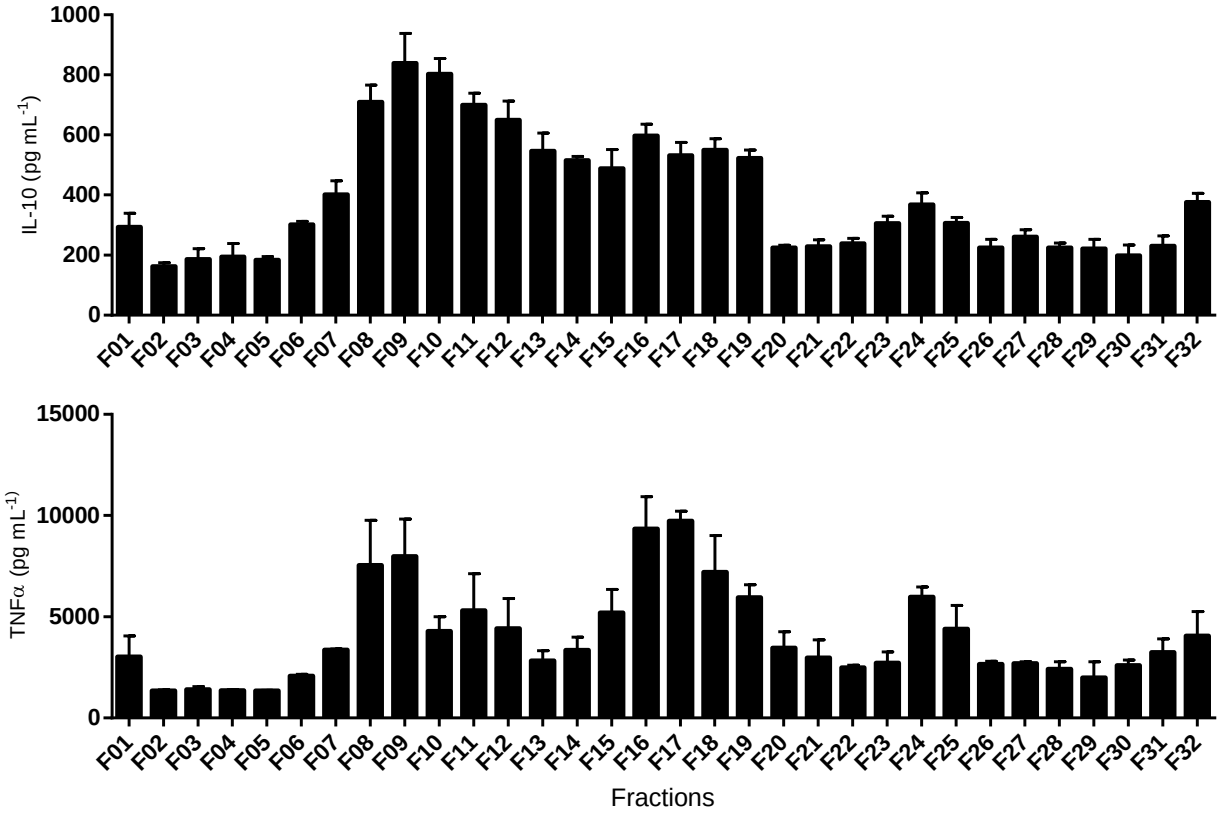

Figure 4.4: Cytokine responses in human PBMCs stimulated with cell wall preparations fractionated using size exchange chromatography. Fractions (F01 to F32) were filtered for sterility and diluted 1:10 to treat human PBMCs for 18 hours. IL-10 (top) and TNF (bottom) accumulation was analyzed as in previous experiments.

High IL-10 levels were observed in fractions 9-12, with peak IL-10 production observed in fraction 9. Fractions 10-15 were identified based on their induced cytokine profiles, showing relatively high IL-10 induction, while inducing only modest levels of TNF (Figure 4.4). Because size-exclusion chromatography does not involve a separate loading and elution phase, the proteins in the cell wall fraction are greatly diluted in the eluted fractions. To test these fractions further, fractions 10-15 were concentrated to test for cytokine responses relative to protein concentration.

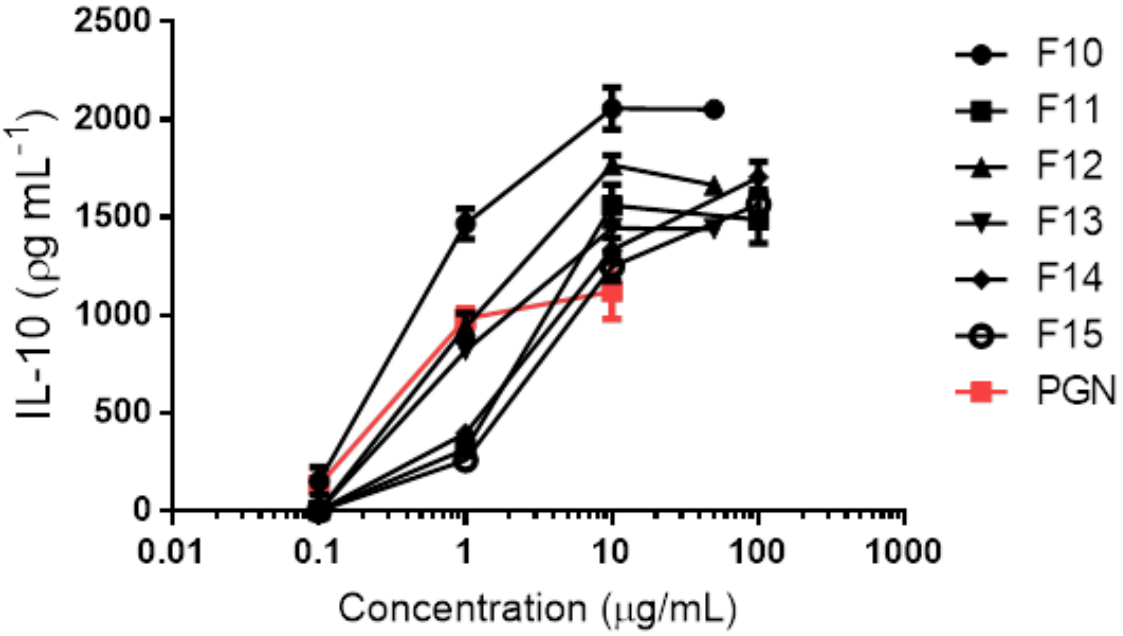

Figure 4.5: IL-10 response in humans PBMCs stimulated with concentrated size-exclusion fractions. Fractions 10-15 were concentrated using centrifuge filters with a 30kDa cut-off, quantified for protein content by BCA, and used to treat human PBMCs at different doses to generate a dose-response curve for cytokine production in response to fraction concentration. PGN was diluted in accordance to manufacturer protocols and used to stimulate PBMCs as a positive control for cytokine accumulation (red line).

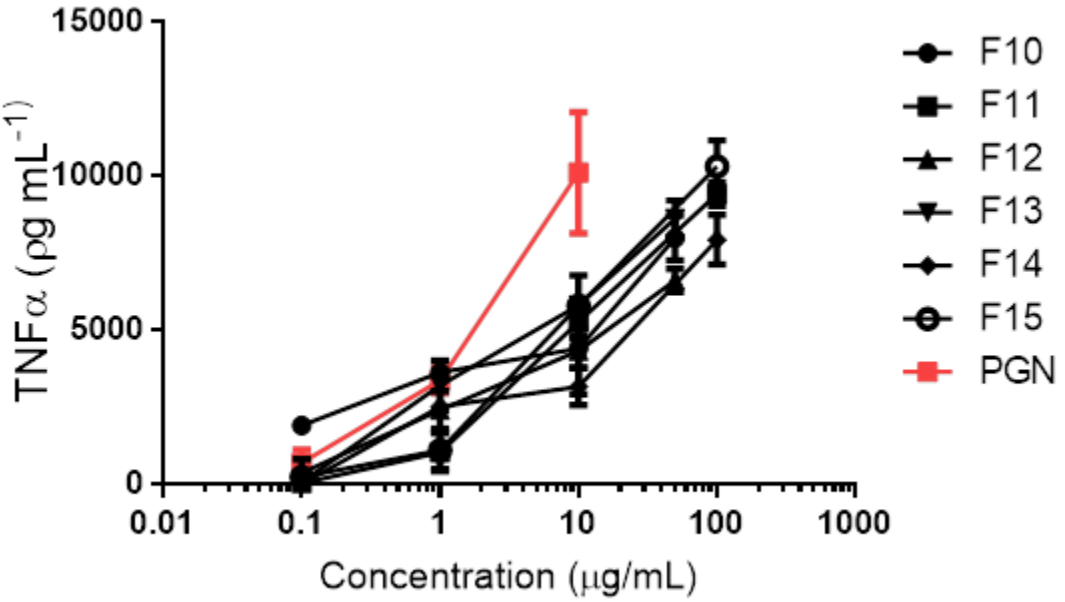

Figure 4.6: TNF response in humans PBMCs stimulated with concentrated size-exclusion fractions. Fractions 10-15 were concentrated using centrifuge filters with a 30kDa cut-off, quantified for protein content by BCA, and used to treat human PBMCs at different doses to generate a dose-response curve for cytokine production in response to fraction concentration. PGN was also used to stimulate PBMCs as a positive control for cytokine accumulation (red line).

In comparison with PGN, the positive control for *S. aureus* cell wall, the concentrated fractions were able to induce a stronger IL-10 and weaker TNF dose-response curves (Figures 4.5 and 4.6). At lower concentrations, PGN induced a stronger IL-10 response compared to some of the fractions. However at the 10 µg/mL concentration, all fractions induced higher IL-10 levels and lower TNF levels compared to the PGN control. This suggested that size-exclusion chromatography generated fractions which were enriched in the IL-10-inducing ligand(s).

4.1.5 *The IL-10-inducing ligand(s) are negatively charged at pH 8 and can be enriched by anion-exchange chromatography*

As previously mentioned, the methodology to prepare the sample for size-exclusion chromatography greatly dilutes the cell wall preparations, creating problems for downstream analyses such as *ex vivo* cell stimulation and further purification procedures. In order to circumvent this problem, ion-exchange chromatography was employed to fractionate the cell wall preparations. Because ion-exchange chromatography is based on affinity between the loaded sample and the stationary phase, the loading phase was separated from the elution phase. This allowed an overall greater amount of material to be loaded, since there were no constraints on the volume of cell wall that can be loaded. In addition, the eluted fractions were concentrated by the elution process.

Ion-exchange chromatography uses electrostatic interactions between the sample components and the column resin to bind to molecules of interest [134]. Since bacterial

cell walls are known to be slightly negatively charged [135], a resin conjugated to positively-charged quaternary ammonium ions was chosen (Q- Sepharose). After loading the cell wall preparation onto the column, fractions were eluted using increasing salt concentrations to outcompete the electrostatic interactions between the stationary phase and proteins of interest and liberate them for elution. UV absorbance at 280 nm was recorded throughout the run.

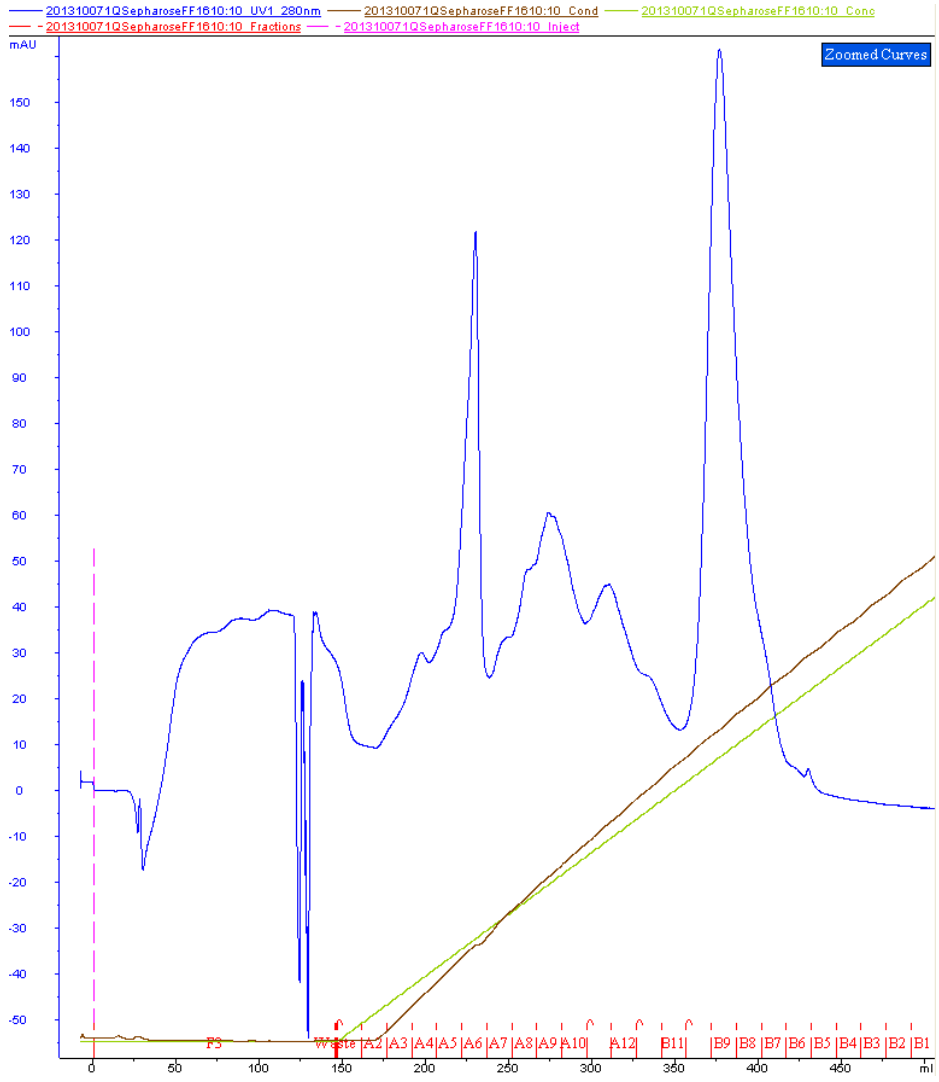

Figure 4.7: Chromatogram of cell wall fractionation using anion-exchange. Cell wall preparations were loaded onto Q-Sepharose resin and eluted using increasing salt concentrations. UV absorbance at 280 nm was recorded to visualize proteins eluting from the column. Lines: Blue (absorbance at 280 nm, aromatic amino acids), Green (salt concentration), Brown (conductivity, reflection of salt concentration). The x-axis tracks the volume of liquid movement through the column with the red markings demarcating the fractions collected, while absorbance at 280nm is shown on the y-axis.

The fractions generated by anion-exchange (Figure 4.7) were quantified for protein content and used to stimulate human PBMCs for cytokine production. The ELISA results indicated that all 20 of the fractions induced IL-10 to varying degrees, making it difficult to determine if any fractions were exception with regard to its activation of the anti-inflammatory response. The cytokine data were normalized to reflect the amount of IL-10 secreted relative to the amount of the fraction used for each dilution. This normalization was done because the anion-exchange was not of sufficiently high resolution, and numerous proteins were eluted in each fraction. If dose-responses were observed from protein concentration alone, co-elution of the hypothesized immunomodulatory component(s) with other abundant proteins would obscure the cytokine response, lowering the signal to noise ratio. Following this normalization, fractions 4 and 5 were observed to induce high levels of IL-10 relative to protein concentration (Figure 4.8). A strong IL-10 dose-response was observed with the cell wall preparation, but only background levels of IL-10 was observed in the column run-through. Fraction 4 showed a high IL-10 response at the highest concentration but modest levels in the lower concentrations. Fraction 5 showed a strong dose-response curve, but the IL-10 induction dropped off at the highest concentration, which is consistent with previous observations (Figure 4.1)

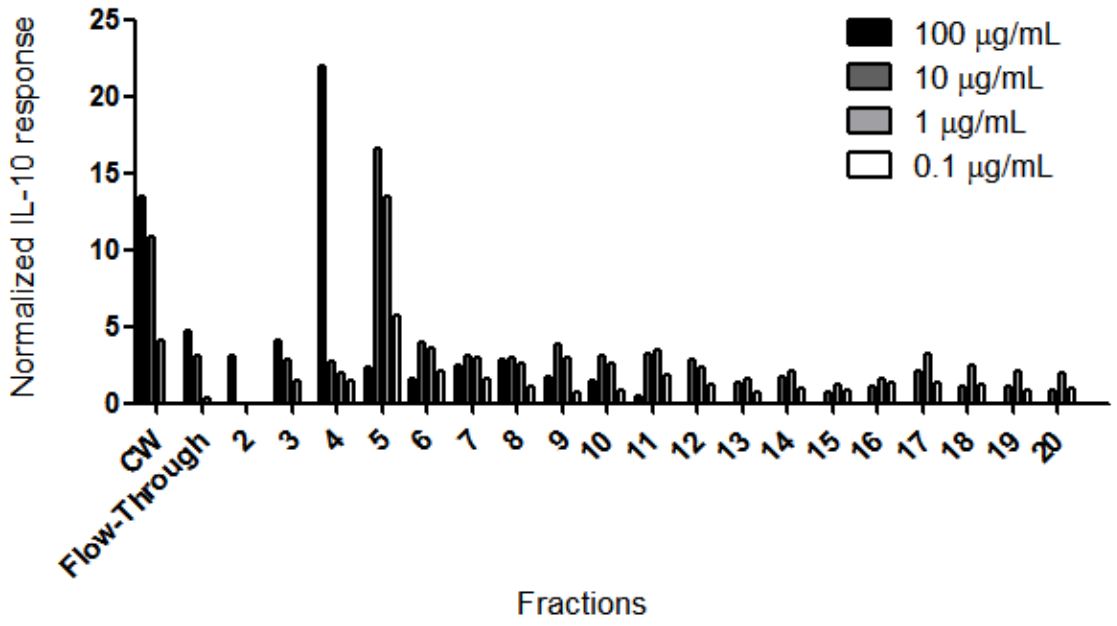

Figure 4.8: **Normalized IL-10 response in human PBMCs stimulated with cell wall preparations fractionated using anion-exchange chromatography**. The protein content of each fraction was measured by BCA assay, and each fraction was diluted to generate dose-response curves. IL-10 responses were normalized by dividing the cytokine response as measured by ELISA by the volume of fraction required to make each dilution. CW indicates the IL-10 observed in cells stimulated with the starting cell wall preparation before anion-exchange.

Anion-exchange was shown to be able to enrich fractions with IL-10 inducing activity (Figure 4.8). In order to generate fractions of higher purity, two-step anion-exchange was performed to separate components of the cell wall by charge with higher resolution. A second round of anion-exchange was undertaken, repeating the Q-Sepharose separation as done previously, and applying fractions of interest to a smaller, higher resolution column. Fractions observed to induce high levels of IL-10 in the first anion-exchange experiment were collected, desalted, and applied to a smaller column packed with Mono-Q resin. This smaller analytical column allows separation into more fractions with a smaller gradient of salt concentrations, yielding higher resolution of separation.

For the first step, cell wall preparations were separated using the same column as previously used (Q-Sepharose). UV absorbance at 220 nm (all amino acids) and 280 nm (aromatic amino acids) were recorded during the chromatography run.

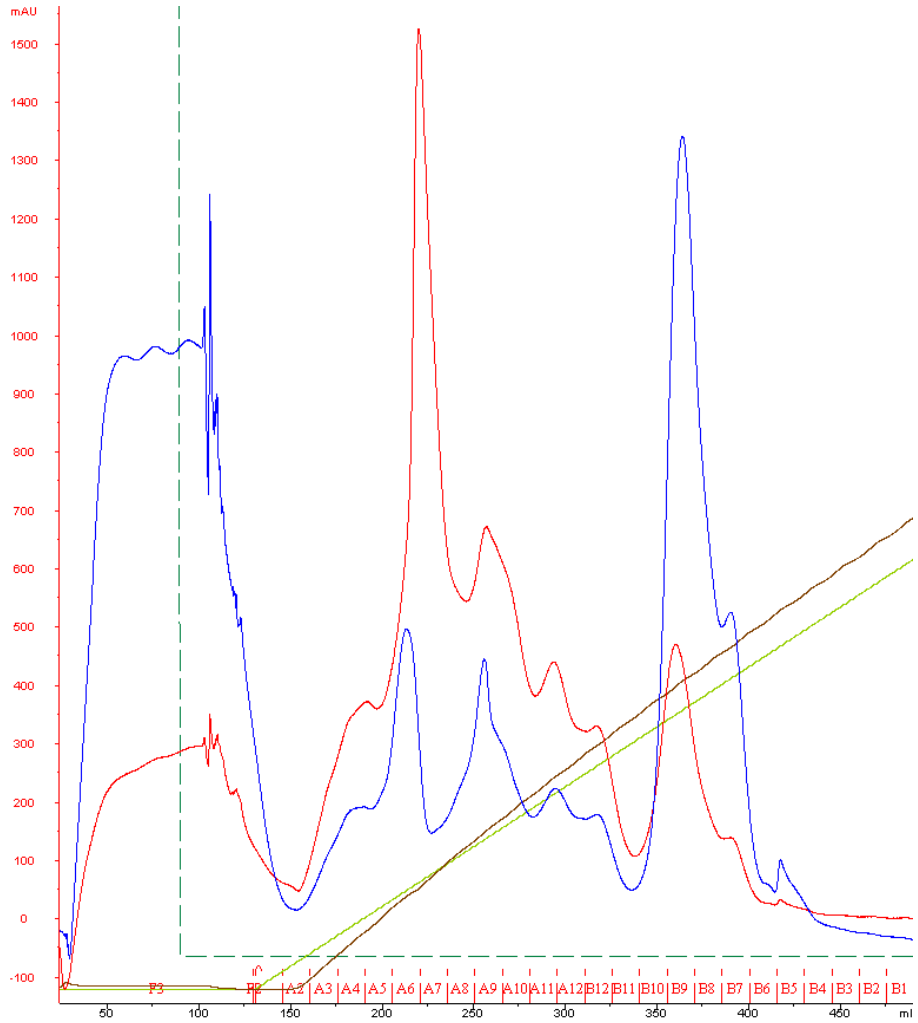

Figure 4.9: Chromatogram of Q-Sepharose anion-exchange chromatography on cell wall preparations. Cell wall preparations were loaded onto Q-Sepharose resin and eluted using increasing salt concentrations. UV absorbance at 220 nm and 280 nm was recorded to visualize proteins eluting from the column. Lines: Blue (absorbance at 280 nm, aromatic amino acids), Red (absorbance at 220 nm, amino acids), Green (salt concentration), Brown (conductivity, reflection of salt concentration). Fractions 3 to 8 were pooled, desalted, and applied to the Mono-Q column (Figure 4.12). The x-axis tracks the volume of liquid movement through the column with the red markings demarcating the fractions collected, while absorbance at 280nm is shown on the y-axis.

The fractions generated by this first step of fractionation (Figure 4.9) were used to stimulate human PBMCs and IL-10 and TNF cytokine responses were examined. Unlike the first anion-exchange run, the protein content of each fraction were not quantified since the overall IL-10-inducing activity of each fraction is of interest, not the total protein content in each fraction. If for example, the IL-10 inducing ligands co-eluted with a highly abundant cell wall protein which is inert for cytokine induction, measuring the specific cytokine response (i.e., diluting all fractions to the same protein concentration) would mistakenly suggest that the fraction is low in IL-10 activity, since the presence of the highly abundant protein would result in the fraction being highly diluted. Measuring cytokine levels by stimulating with all fractions using the same dilution scheme would identify which fractions induce the highest cytokine levels overall. PBMCs were stimulated with Q-Sepharose fractions which were diluted 1:100, 1:1000, and 1:10000.

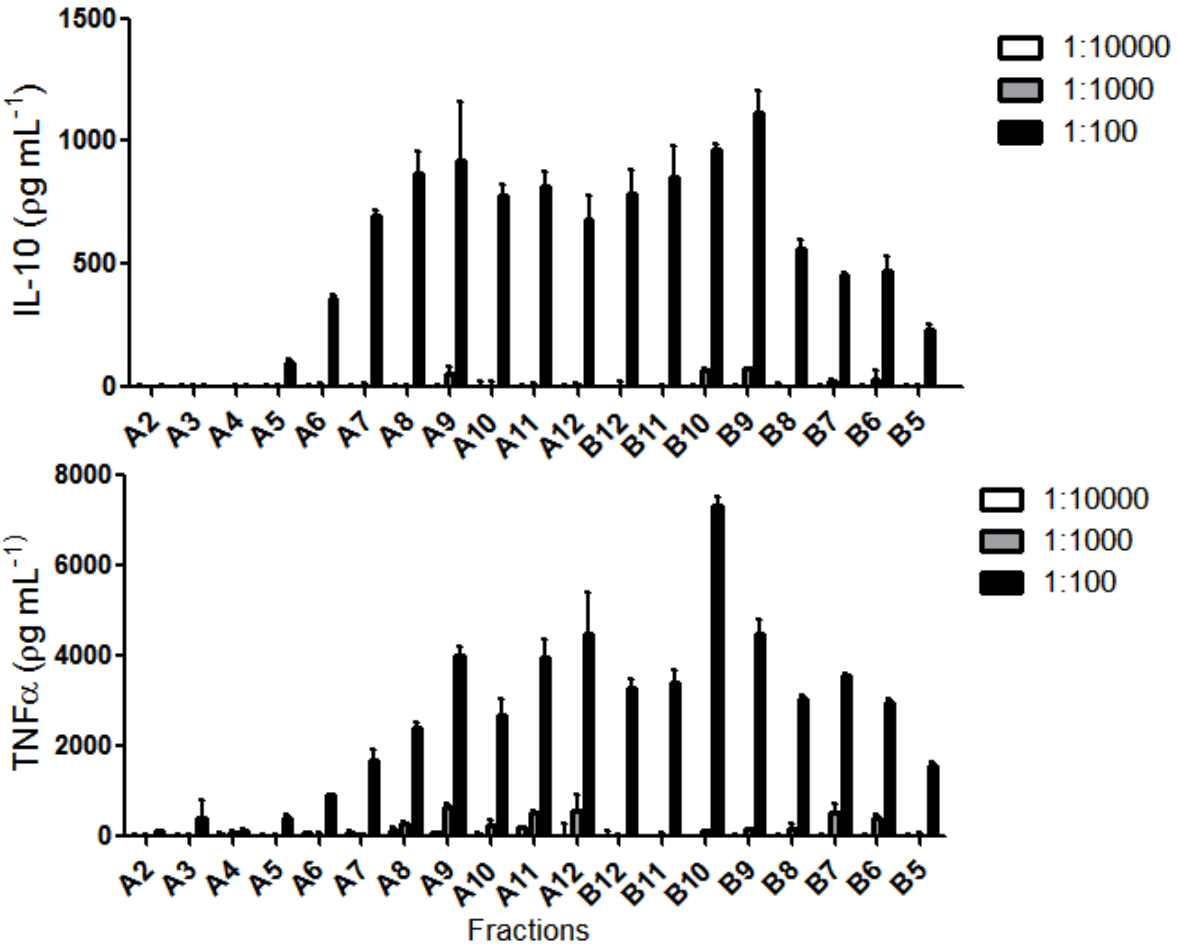

Figure 4.10: Cytokine responses in human PBMCs stimulated with cell wall preparations fractionated by anion-exchange chromatography using Q-Sepharose resin. Cell wall fractions were diluted 1:100, 1:1000, and 1:10000 and used to stimulate human PBMCs. Supernatant IL-10 (top) and TNF (bottom) accumulation were determined by ELISA as before.

On the second run of anion-exchange using the Q-Sepharose resin, the IL-10 response was observed to be widely induced by many fractions (Figure 4.10). Since the objective was to identify fractions that preferentially induced IL-10 over TNF, the IL-10 to TNF ratio was calculated for each fraction at the 1:100 dilution, which gave cytokine responses in all fractions (Figure 4.11). If the hypothesis that a distinct set of ligands gives rise to the pro- and anti-inflammatory cytokine responses is correct, fractions enriched in IL-10 activity should exhibit a higher IL-10 to TNF ratio compared to the cell wall preparation applied to the column.

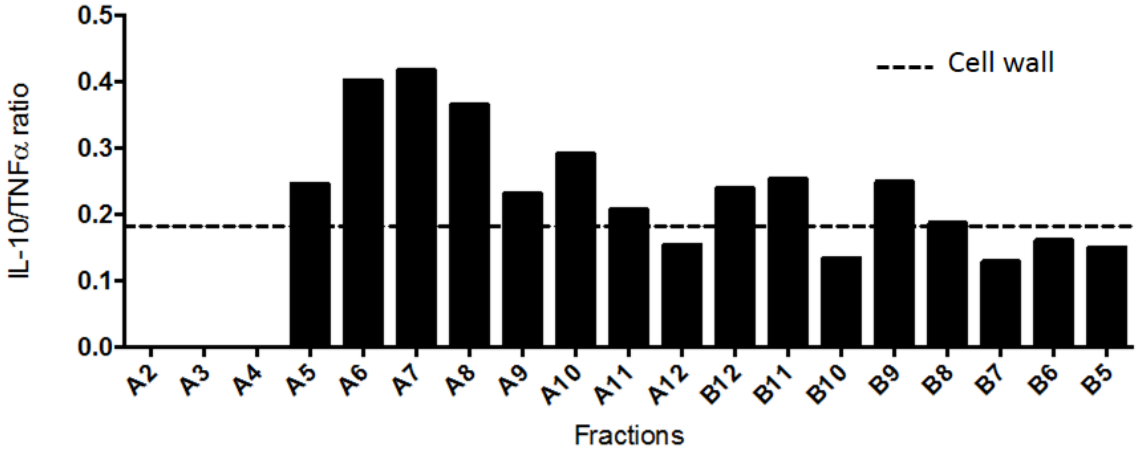

Figure 4.11: IL-10 to TNF ratio observed in human PBMCs stimulated with Q-Sepharose fractions at 1:100 dilution. Cytokine responses from Figure 4.10 were used to calculate the IL-10 to TNF ratio, since fractions containing specific IL-10-inducing ligands would be expected to enhance the IL-10 to TNF ratio. The dashed line represents the cytokine response to the cell wall preparation.

Fractions 6-8 showed a high induction of IL-10 relative to levels of TNF (Figure 4.11). These fractions exhibited approximately double the IL-10 to TNF ratio compared to the ratio of the cell wall preparation. No IL-10 induction capacity was observed for fractions 2-4. IL-10 to TNF ratios slightly above the cell wall control was observed for other fractions (A10, B12, B11, B9), but with a lower magnitude compared to fractions 6-8. Thus, fractions 6-8 were prioritized for downstream purification. In order to maximize the yield of anti-inflammatory compounds, fractions 3-8 were pooled and fractionated again on the Mono-Q analytical anion-exchange resin. As previously, UV absorbance at 220 nm (all amino acids) and 280 nm (aromatic amino acids) were recorded during the chromatography run (Figure 4.12).

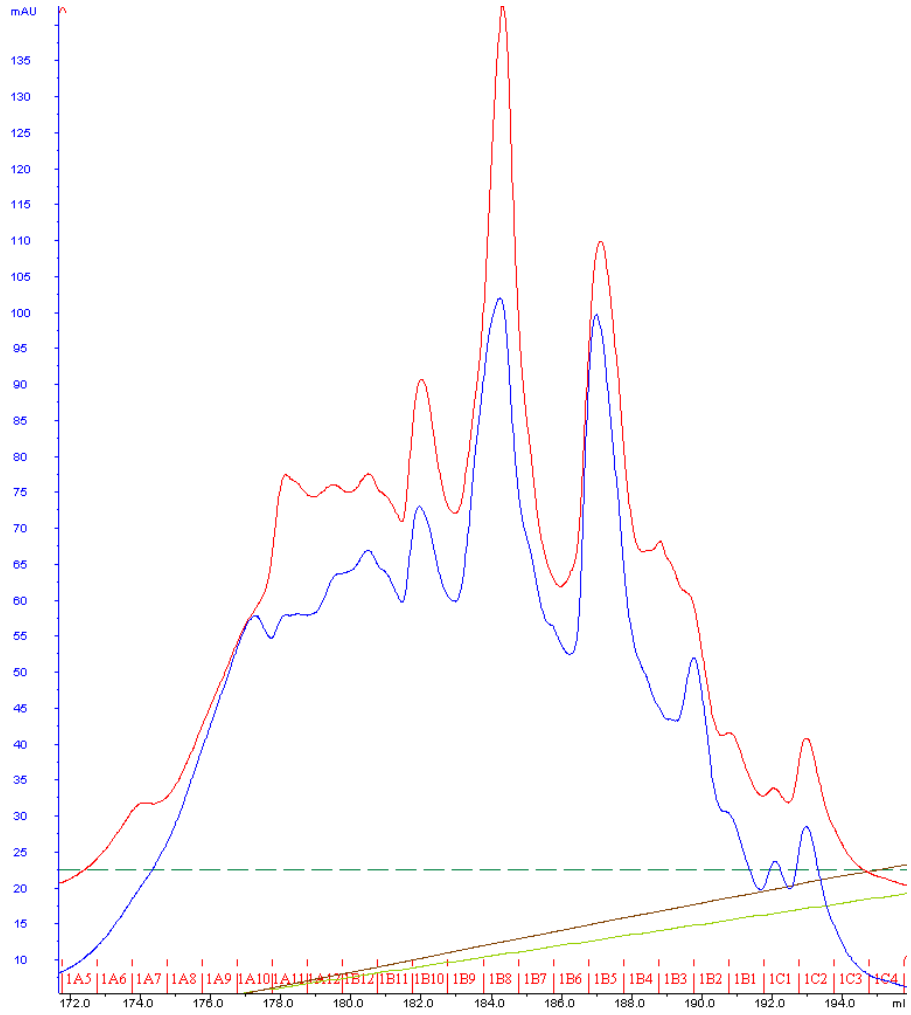

Figure 4.12: Chromatogram of Mono-Q anion-exchange chromatography on fractions 3-8 of the Q-Sepharose run. Fractions 3-8 of the Q-Sepharose run were pooled, desalted, and loaded onto Q-Sepharose resin and eluted using increasing salt concentrations. UV absorbance at 220 nm and 280 nm was recorded to monitor proteins eluting from the column. Lines: Blue (absorbance at 280 nm, aromatic amino acids), Red (absorbance at 220 nm, amino acids), Green (salt concentration), Brown (conductivity, reflection of salt concentration). The x-axis tracks the volume of liquid movement

through the column with the red markings demarcating the fractions collected, while absorbance at 280nm is shown on the y-axis.

The chromatogram of the second anion-exchange run with the Mono-Q resin on fractions 3 to 8 showed that the cell wall preparation was heterogenous, with many peaks in the UV absorbance. Fractions 3-8 from the Q-Sepharose chromatogram appears as one large peak with a shoulder, but resolved into two large peaks with many smaller peaks on the Mono-Q resin. The Mono-Q fractions were used to stimulate humans PBMCs for cytokine production.

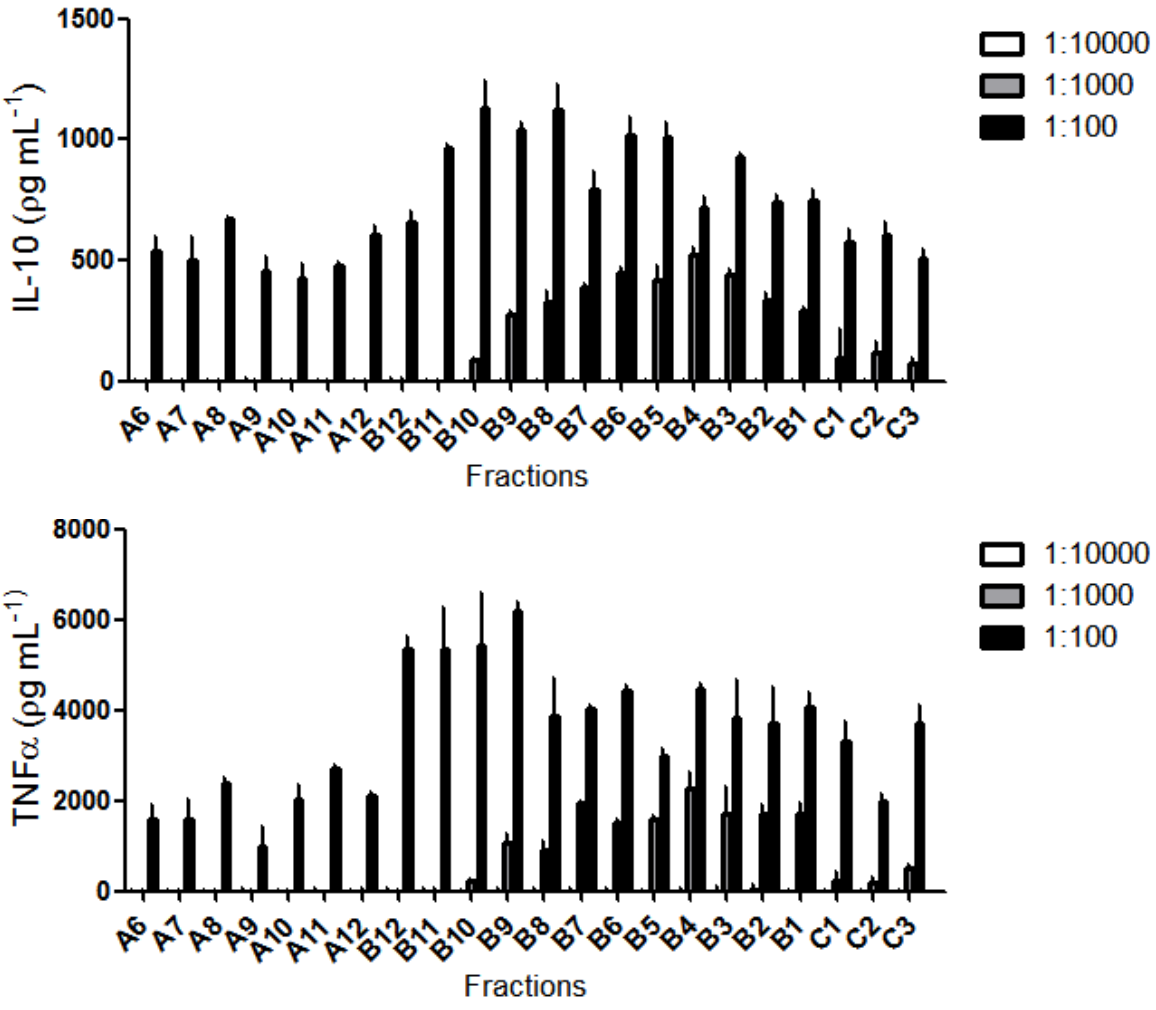

Figure 4.13: Cytokine responses in human PBMCs stimulated with Mono-Q fractions. Fractions 3-8 from the Q-Sepharose fractionation were further resolved using the Mono-Q resin. Fractions were diluted and used to stimulate human PBMCs to assess cytokine responses by ELISA as before.

As was observed with the cytokine responses following stimulation with the Q-Sepharose fractions, the IL-10-inducing activity after PBMC stimulation using Mono-Q fractions was spread across many fractions (Figure 4.13). Since no clear patterns were observed by looking at the raw cytokine concentration data, the IL-10 to TNF ratio was calculated to look for enrichment of IL-10 inducing capacity.

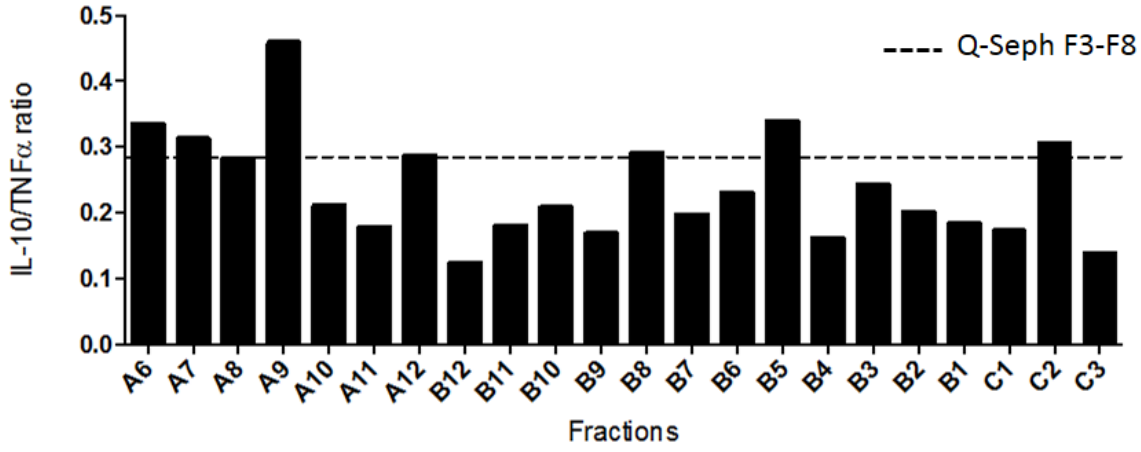

Figure 4.14: IL-10 to TNF ratio observed in human PBMCs stimulated with Mono-Q fractions at 1:100 dilution. Cytokine responses from Figure 4.13 were used to calculate the IL-10 to TNF ratio, since fractions containing specific IL-10-inducing ligands would be expected to enhance the IL-10 to TNF ratio. Dashed line represents the starting material (i.e. Q-Sepharose fractions 3-8 which was desalted).

When the IL-10 to TNF ratio was plotted for the Mono-Q fractions, no clear patterns where observed for fractions which clearly preferentially induced IL-10 (Figure 4.14). Fraction 9 exceeds the IL-10 to TNF ratio of the starting material, but this was not observed with the flanking fractions, as was observed with fractions 6-8 of the Q-Sepharose run (Figure 4.11). Since fraction 9 was not associated with a clear peak in the chromatogram (Figure 4.12), this is likely an artifact. A single fraction that enhances the IL-10 to TNF ratio is explainable if the fractionation was sufficiently high resolution to elute a protein in a single fraction (i.e. a single, sharp peak contained in the volume of a single fraction). However, the chromatogram suggests that this level of resolution was not achieved, as peaks are generally wider than the width of a fraction as seen in the chromatograms.

The spread of IL-10-inducing activity across both the Q-Sepharose and the Mono-Q fractions confirmed the high level of heterogeneity present in the cell wall preparations (Figures 4.11, 4.13). In order to visualize the components of the cell wall, Q-Sepharose fractions were visualized by SDS-PAGE and silver staining. When Q-Sepharose fractions were subjected to denaturing conditions and separated by SDS-PAGE, a large number of components in each fraction was observed (Figure 4.15).

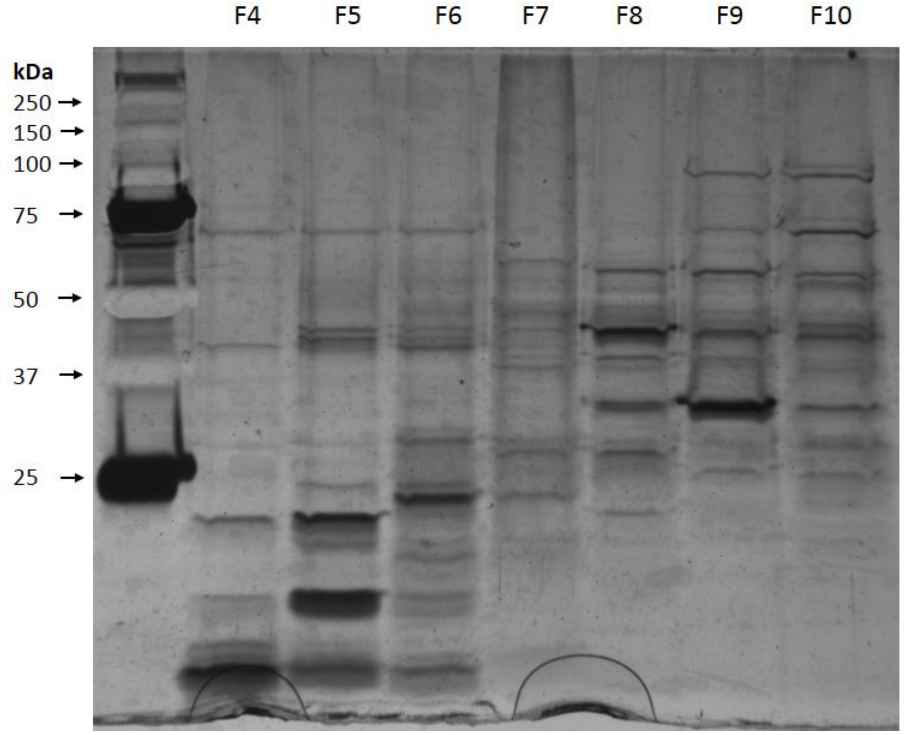

Figure 4.15: Q-Sepharose fractions 4-10 are highly heterogenous mixtures of proteins. Fractions 4 to 10 were denatured by the addition of SDS sample buffer and boiling. They were run on a 10% polyacrylamide gel and visualized by silver staining.

4.1.6 *Proteomics of Q-Sepharose cell wall fractions identified proteins involved in S. aureus virulence*

While higher resolution of separation was achieved with the Mono-Q resin in terms of the chromatogram (i.e. more peaks observed), the additional Mono-Q step following the Q-Sepharose separation yielded no resolving power for the ability to preferentially induce IL-10, since none of the Mono-Q fractions were convincingly enriched in IL-10 to TNF ratio compared to the starting material (ie. pooled fractions 3-8 from the Q-Sepharose separation). To examine the molecular makeup of fractions that were highly enriched in IL-10 activity, fractions 6-8 of the Q-Sepharose separation were pooled and sent to the proteomics platform of the Institute for Research in Immunology and Cancer at Université de Montréal. Given the sensitivity inherent in mass spectrometry, hundreds of proteins were detected by the mass spectrometer. After narrowing down the hits to proteins which were larger than approximately 50 kDa and that were anchored on the cell wall, several entries stood out as being particularly interesting (Table 4.1). These proteins shared two features. First, all of these proteins had functions which correlate with *S. aureus* pathogenicity, as many are involved with binding to components of the host extracellular matrix, while others are involved in coagulating plasma [136], discussed previously as a mechanism for evading immune surveillance. Second, all of the tabulated proteins require the action of the staphylococcal enzyme Sortase A for anchorage to the cell wall [137].

Table 4.1: Sortase A targets identified in a proteomics analysis of Q-Sepharose fractions. Proteomics targets were trimmed to exclude proteins of less than approximately 50 kDa and were not anchored to the cell wall. The following proteins were manually selected as proteins which were involved in *S. aureus* pathogenesis. The protein score represents the overall level of certainty given the number of peptides and concordance between amino acid sequences detected and database peptides.

Protein ID	Protein Description	Protein Mass	Score
gi|49240921	bone sialoprotein-binding protein [Staphylococcus aureus subsp. aureus MRSA252]	49101	724
gi|49243016	collagen adhesin precursor [Staphylococcus aureus subsp. aureus MRSA252]	132968	348
gi|49241173	clumping factor [Staphylococcus aureus subsp. aureus MRSA252]	106655	291
gi|49245914	collagen adhesin precursor [Staphylococcus aureus subsp. aureus MSSA476]	132986	128
gi|397526	clumping factor [Staphylococcus aureus]	97002	94
gi|14246330	Ser-Asp rich fibrinogen-binding; bone sialoprotein-binding protein [Staphylococcus aureus subsp. aureus Mu50]	142691	46
gi|49240921	bone sialoprotein-binding protein [Staphylococcus aureus subsp. aureus MRSA252]	127048	36
gi|57867844	cell wall associated fibronectin-binding protein [Staphylococcus epidermidis RP62A]	1050153	32
gi|88196585	clumping factor B; putative [Staphylococcus aureus subsp. aureus NCTC 8325]	97191	30

4.1.7 *The IL-10 response of S. aureus is dependent on Sortase A, a cell wall anchoring enzyme*

Sortase A was discovered in 1999 as mutants deficient in Sortase A were unable to anchor certain proteins to the cell wall [137]. It was found to be highly conserved in *S. aureus* strains, and all Gram-positive bacteria were found to carry a homolog of Sortase [137]. Sortase A catalyzes a transpeptidation reaction which attaches proteins carrying the conserved LPXTG motif to the cell wall.

Since proteins requiring Sortase A for cell wall targeting were found in the proteomics analysis, Sortase A deficient *S. aureus* were assayed directly for IL-10 induction. *S. aureus* deficient in Sortase A were grown in the same conditions as the previously used *S. aureus* community isolates, and used to stimulate human PBMCs for cytokine induction. The Sortase A-deficient *S. aureus* was found to have drastically decreased levels of IL-10 induction compared to the parental wild-type strain (Figure 4.16).

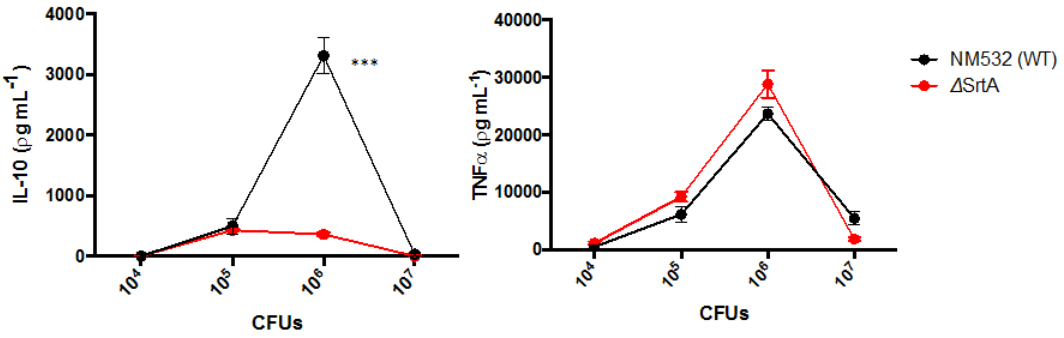

Figure 4.16: IL-10 and TNF responses of human PBMCs stimulated with either wild-type (WT) or Sortase A-deficient (*ΔSrtA*) *S. aureus* for 18 hours. Wild-type and Sortase A deficient strains were grown in identical conditions and bacterial densities were determined by CFU counting. Human PBMCs were stimulated with the indicated amounts of bacteria for 18 hours and supernatants were collected for cytokine analysis by ELISA. *** indicates $p < 0.001$

4.1.8 *Deficiency in proteins with a Sortase A targeting sequence affects IL-10 responses*

The inability of the Sortase A-deficient *S. aureus* to induce IL-10 suggested that the presence of Sortase A is required for IL-10 induction, either directly or through its enzymatic action. Sortase A recognizes an amino acid motif which targets proteins for cell wall anchorage: LPXTG [137]. From bioinformatics analyses, 20 proteins from *S. aureus* were found to carry this motif, and were identified as proteins which were dependent on Sortase A for conjugation to the cell wall. A library of *S. aureus* mutants were obtained from the Nebraska Transposon Mutant Library (NTML). This library, comprising approximately 2000 viable strains with a single gene knocked out by insertion [138], is generated by random insertion of a transposon into the *S. aureus* genome of a parental strain, with the insertion locations ascertained by sequencing [138]. Eight proteins known to be dependent on Sortase A have knock-outs represented in the NTML. These strains were grown, and used to stimulate human PBMCs for cytokine responses. Since the parental strain was not included in the library for use as a control, a gene mutant which is not expected to contribute to cell wall components was included as a control (MutL, involved in DNA mismatch repair) [139].

Puzzlingly, only the Sortase A deficient strain displayed the expected phenotype of decreased IL-10 induction (Figure 4.17). For the Sortase A targets, most showed similar patterns of IL-10 induction as the MutL control (fnbA, fnbB, clfB). These proteins are involved in blood clotting (clumping factor B; clfB) and fibronectin adhesion (fnbA,

fnbB) [140], and their deficiency was not associated with any change in cytokine induction. Unexpectedly, some strains displayed enhanced IL-10 induction, and induced greater IL-10 with less bacteria (LPXTG-motif containing protein, clfA, SdrC, SdrD, and SdrE). ClfA is involved in blood clotting (clumping factor A; clfA) [140], SdrC, SdrD, and SdrE are involved in binding to fibrinogen and sialoproteins [141].

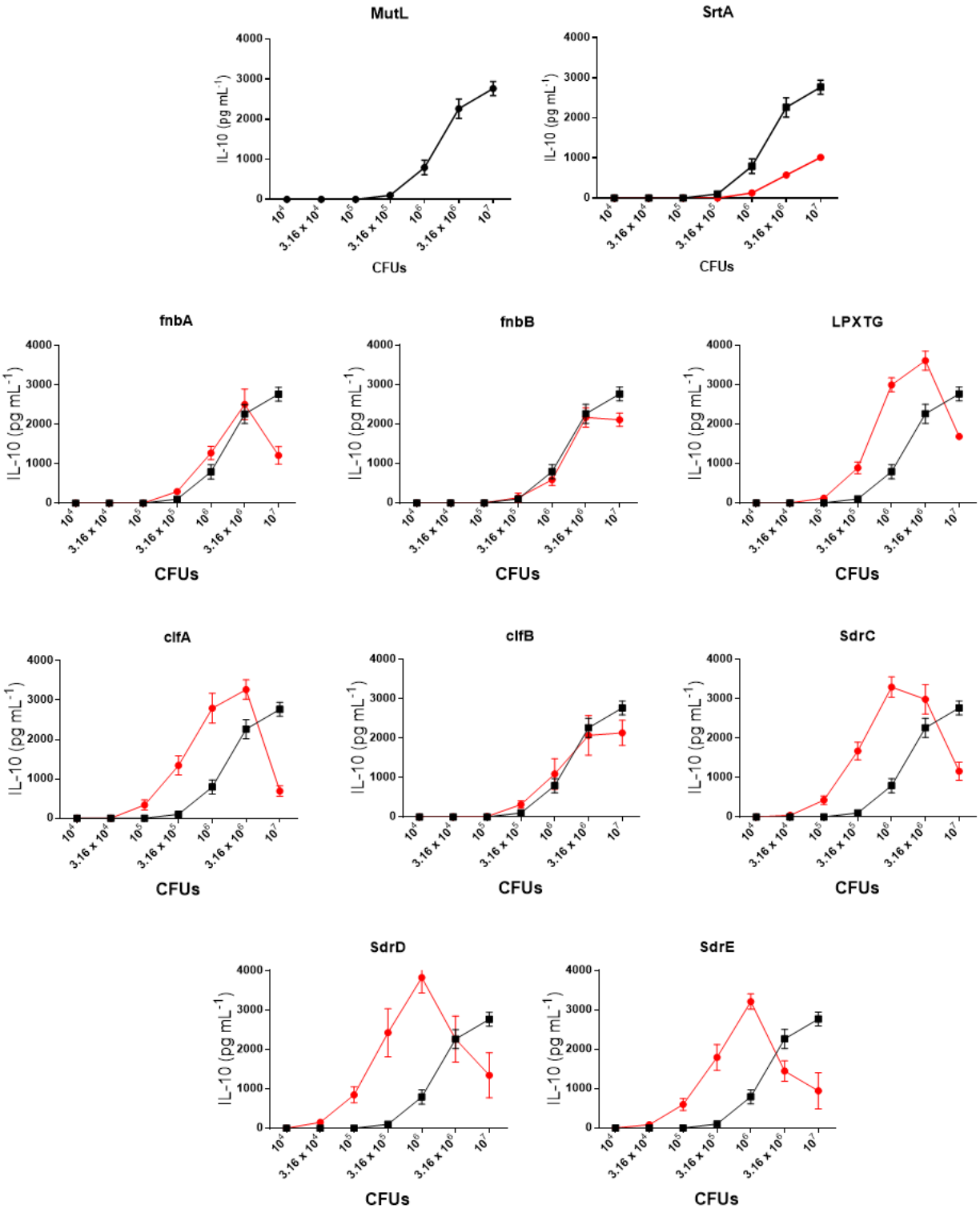
MutL
SrtA
fnbA
fnbB
LPXTG
clfA
clfB
SdrC
SdrD
SdrE
IL-10 (pg mL-1)
CFUs
4000
3000
2000
1000
0
10^4
3.16 x 10^4
10^5
3.16 x 10^5
10^6
3.16 x 10^6
10^7

Figure 4.17: Cytokine responses in human PBMCs stimulated with varying colony-forming units (CFU) of bacteria deficient in Sortase A or Sortase A targets. A sortase A deficient strain, along with other proteins in the NTML library which requires Sortase A for cell wall anchoring were grown, counted, and used to stimulate human PBMCs for 18 hours. Supernatants were collected for cytokine response analysis by ELISA. MutL serves as a control, and is reproduced in each plot by the black line, while the deficient strains are represented by the red lines.

4.2 ***Specific aim 2: To develop TLR-Fc fusion proteins to study physiological TLR2 ligands***

An ongoing problem in the study of ligands and signaling through TLR2 is the lack of tools which identify ligands in a manner which is not dependent on the purity of the preparations tested. Contamination with undesired molecules has confounded the results of many previous studies [118]. In addition, the use of *in vitro* reporter gene systems biases results towards an assumed outcome (in the case of TLRs, often NFκB activation) [142], disregarding other potential downstream effectors as a consequence of TLR activation. This is particularly important for TLR2 since a number of heterodimeric receptor complexes are possible (TLR2/1, TLR2/6, TLR2/10), giving a plausible basis for differential intracellular signaling [77].

This specific aim attempted to address this problem by generating a system where the affinity to TLR2/1/6/10 and combinatorial receptor complexes can be used as a basis for identifying potential TLR ligands from a complex ligand mixture (i.e. a cell wall preparation). This strategy involved creating fusion proteins which combined the extracellular domain of the TLRs of interest with human IgG Fc domains. Using TLR-Fc fusion proteins to study ligand binding has already been shown in a study which observed that TLR2 binds to acute-phase serum amyloid A protein [143]. The Fc domain is advantageous because the strong interactions between Protein A and Fc domains will allow the use of commercial antibody purification and immunoprecipitation-based

technologies to be used to purify the TLR-Fc proteins and pull-down molecules which interact with these fusion proteins. The immunologically active Fc domain from human IgG1 is able to potentially activate antibody-dependent cellular cytotoxicity [144], presenting intriguing possibilities for its use both as a research and therapeutic tool.

4.2.1 *Construction of TLR-Fc fusion constructs*

In order to fuse TLR ectodomains with IgG1 Fc regions and express the fusion protein in mammalian cells, the pFuse plasmid was used (Figure 4.18). This plasmid features the IgG1 Fc domain, a recombinant viral promoter, an IL-2 secretion sequence, and the *Sh ble* gene, conferring resistance to the antibiotic Zeocin in both bacterial and mammalian expression systems [143].

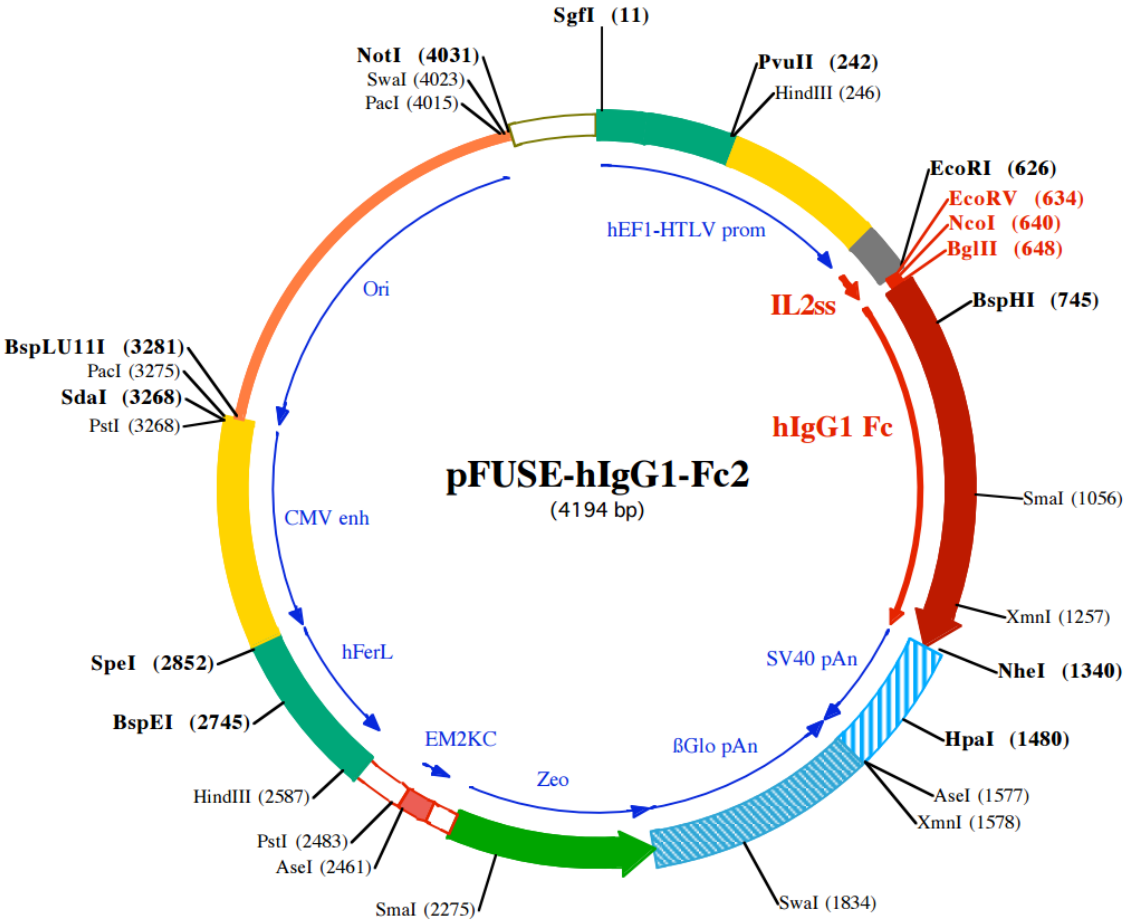

Figure 4.18: A schematic view of the pFuse-hIgG1-Fc2 plasmid This plasmid features a fusion viral promoter, an IL-2 secretion sequence to allow secretion in mammalian cells, and the *Sh ble* gene which confers resistance to the antibiotic Zeocin in both prokaryotic and mammalian expression systems.

DNA encoding for the extracellular domains of TLRs 1, 2, 6, and 10 were amplified by PCR and cloned into the pFuse plasmid using the blunt-end EcoRV site. The plasmids of successful transformants were assessed for the correct size (vector + insert) by restriction enzyme digestion analysis. Colonies which contained plasmids with the insert were sequenced to verify the orientation of the insert since blunt-end cloning does not have orientation specificity. Clones with the insert in the correct orientation and reading frame were obtained for TLRs 1, 2, 6, and 10.

4.2.2 *Assessment of transfection efficiency of a control GFP plasmid by flow cytometry*

TLR-Fc fusion constructs needed to be transfected into a mammalian expression system to produce native state proteins, since TLRs are known to require chaperones for correct folding [145]. The Lonza Nucleofection technology was chosen for transfections, and the optimized solutions and protocol for Chinese Hamster Ovary cells (CHO) were used. CHO cells were seeded in 6 well plates 2 days prior to transfection. On the day of transfection, CHO cells were trypsinized, resuspended in supplemented Nucleofection buffer, and combined with 2 µg of plasmid DNA. The Nucleofection program V-23 was used to electroporate the cells. To assess transfection efficiency, CHO cells were also transfected with the supplied pmaxGFP plasmid. CHO cells were stained with viability dye and examined for transfection efficiency 24 hours post-transfection by flow cytometry (Figure 4.19).

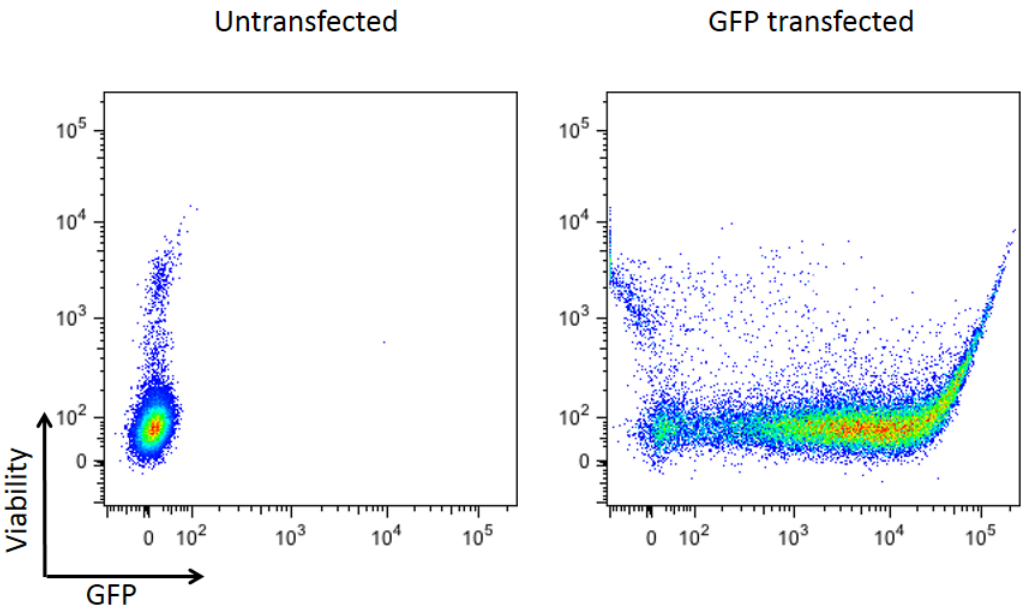

Figure 4.19: Flow cytometry analysis of untransfected and GFP-transfected CHO cells. CHO cells transfected with the pmaxGFP control plasmid were stained with viability dye and analyzed by flow cytometry 24 hours post-transfection. GFP fluorescence is shown on the x axis while the fluorescence of the viability dye (positive indicates cells with permeable cell membranes) is shown on the y axis.

Flow cytometry on CHO cells transfected with the pmaxGFP plasmid indicated that a large proportion of cells (> 80%) expressed the transfected gene 24 hours post-transfection, with low proportions of cell death (< 3%). Forty-eight hours after transfection, Zeocin was added to culture at a concentration of 1 mg/mL to begin selecting for cells which expressed the transfected plasmid carrying the gene conferring resistance to Zeocin. A high concentration of Zeocin was chosen to provide stringent conditions for antibiotic selection of stably transfected clones. The cells resultant from this selection process were termed CHO-TLR-Fc cells.

4.2.3 *Verification of TLR2-Fc expression in CHO-TLR2-Fc lysates*

After transfected CHO cells were selected in the presence of Zeocin for 14 days, cell lysates and supernatants of transfected cells were collected for further assessment of TLR-Fc fusion protein expression. Cell lines transfected with all four fusion constructs have been established, but the verification of TLR2-Fc expression in cell lysates and supernatants was prioritized. CHO-TLR2-Fc cell lysates were obtained and blotted with a polyclonal α-TLR2 antibody. The TLR2-Fc protein was detected at the predicted molecular weight (95 kDa) in the lysate of the transfected CHO-TLR-Fc cells, but not in the untransfected cells (Figure 4.20). Other bands were observed in both the untransfected and transfected cells. Since the antibody used to blot the membrane was polyclonal, these likely are non-specific bands.

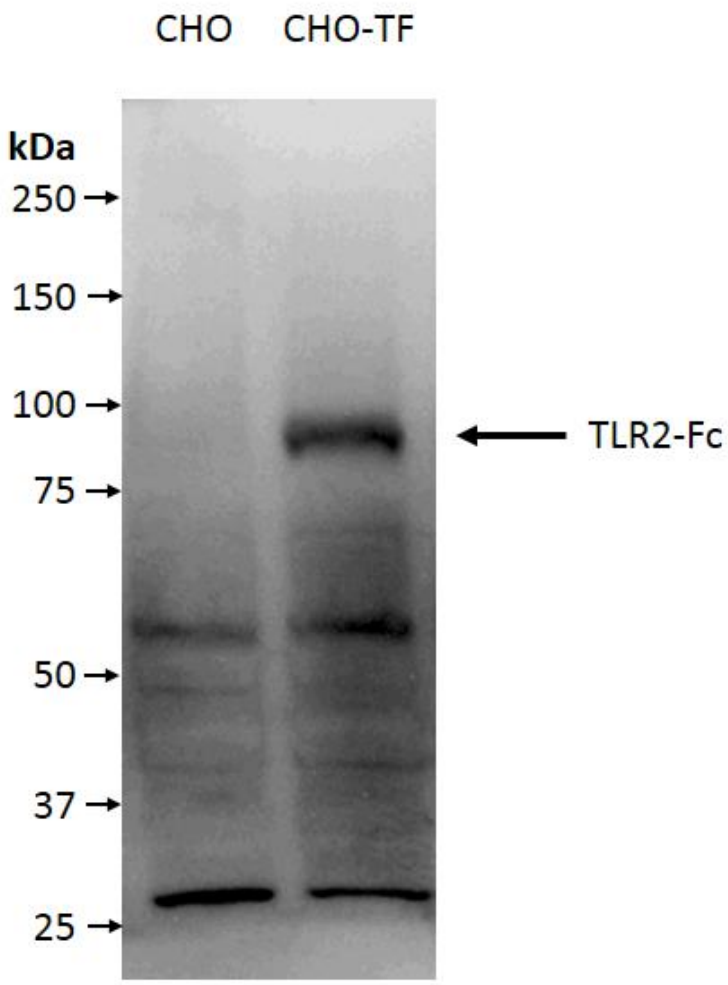

Figure 4.20 Detection of TLR2-Fc expression in transected CHO cells. Untransfected (CHO) and TLR2-Fc transfected CHO cells (CHO-TF) were lysed and the lysates were visualized by SDS-PAGE and western blot for TLR2. The size of the expected TLR2-Fc fusion protein is approximately 95kDa.

4.2.4 *Detection and purification of TLR2-Fc secreted into cell supernatant*

Fourteen days post-transfection, TLR2-Fc protein was detected in the lysates of the transfected CHO-TLR2-Fc cells. However, since the pFuse plasmid contains an IL-2 secretion sequence, the fusion protein is expected to be secreted into the cell culture supernatant. Supernatants were collected, concentrated using a centrifuge filter (>30kDa), and purified using Protein A magnetic beads. The concentrated supernatant was incubated with Protein A magnetic beads for 10 minutes at room temperature, washed 3 times with PBS, and eluted in denaturing conditions by boiling for 10 minutes in SDS sample buffer. The eluted proteins were run on a 10% polyacrylamide gel and blotted for TLR2 using a monoclonal antibody.

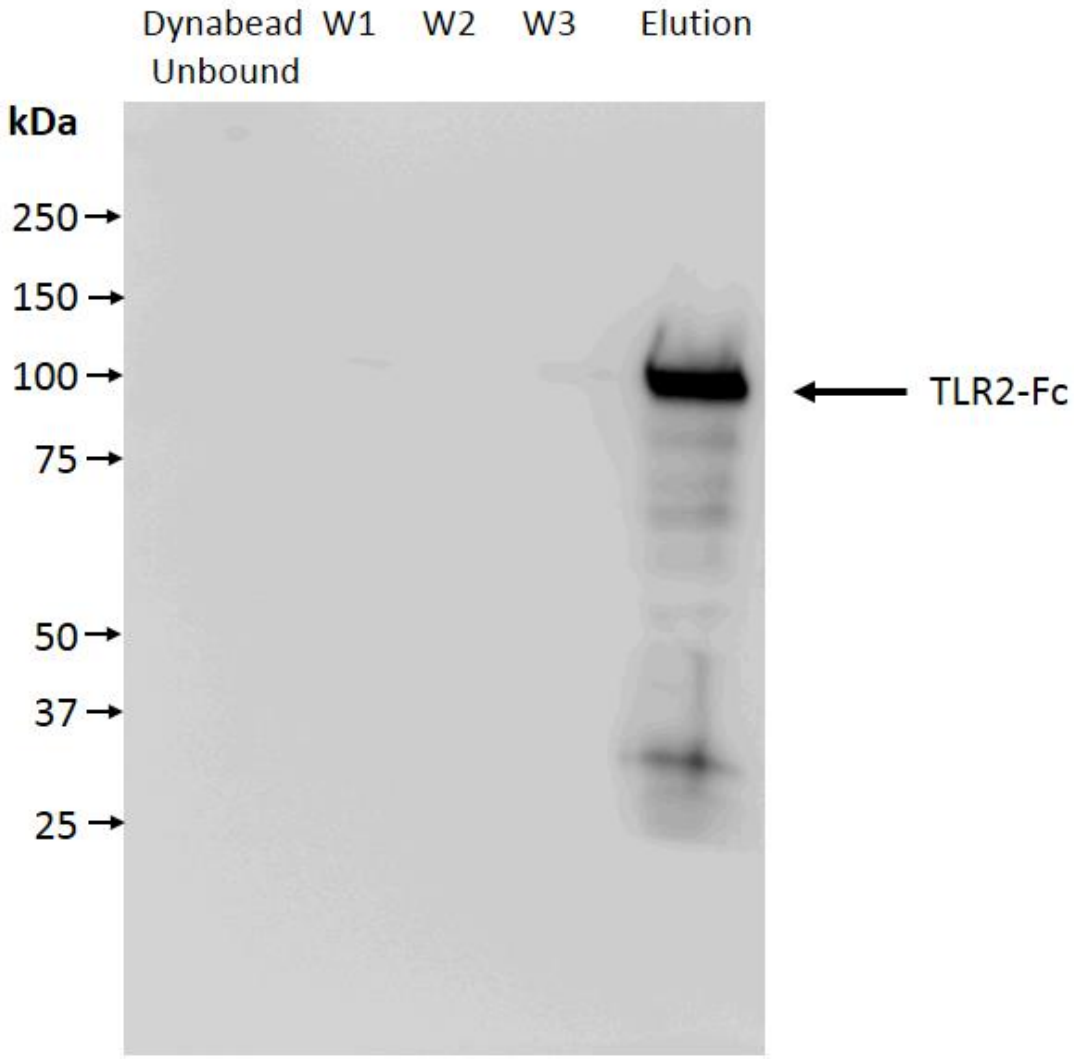

Figure 4.21: Detection of TLR2 in purified CHO-TLR2-Fc supernatant.

Concentrated CHO-TLR2-Fc supernatant was purified using magnetic Protein A beads. The unbound fraction (left over after magnetic bead incubation with concentrated supernatant), 3 washes, and elution were blotted for TLR2. The size of the expected TLR2-Fc fusion protein is approximately 95kDa.

TLR2-Fc protein was detected in the supernatant of the cell culture (Figure 4.21). However, dynabead purification involves elution by boiling the beads in SDS sample buffer (native elution was attempted but was unsuccessful). The action of the SDS (a surfactant) and β-mercaptoethanol (a reducing agent) in the sample buffer denatures the protein as it is being eluted. In order to use the TLR2-Fc protein for functional studies which require its folded conformation for binding, it is necessary to purify the protein in non-denaturing conditions. To do this, Protein A-conjugated agarose columns were used to purify CHO-TLR2-Fc culture supernatants. The purified CHO-TLR2-Fc proteins were eluted using a low pH buffer. TLR2-Fc fusion protein were detected in the elution fractions of the Protein-A column purification (Figure 4.22).

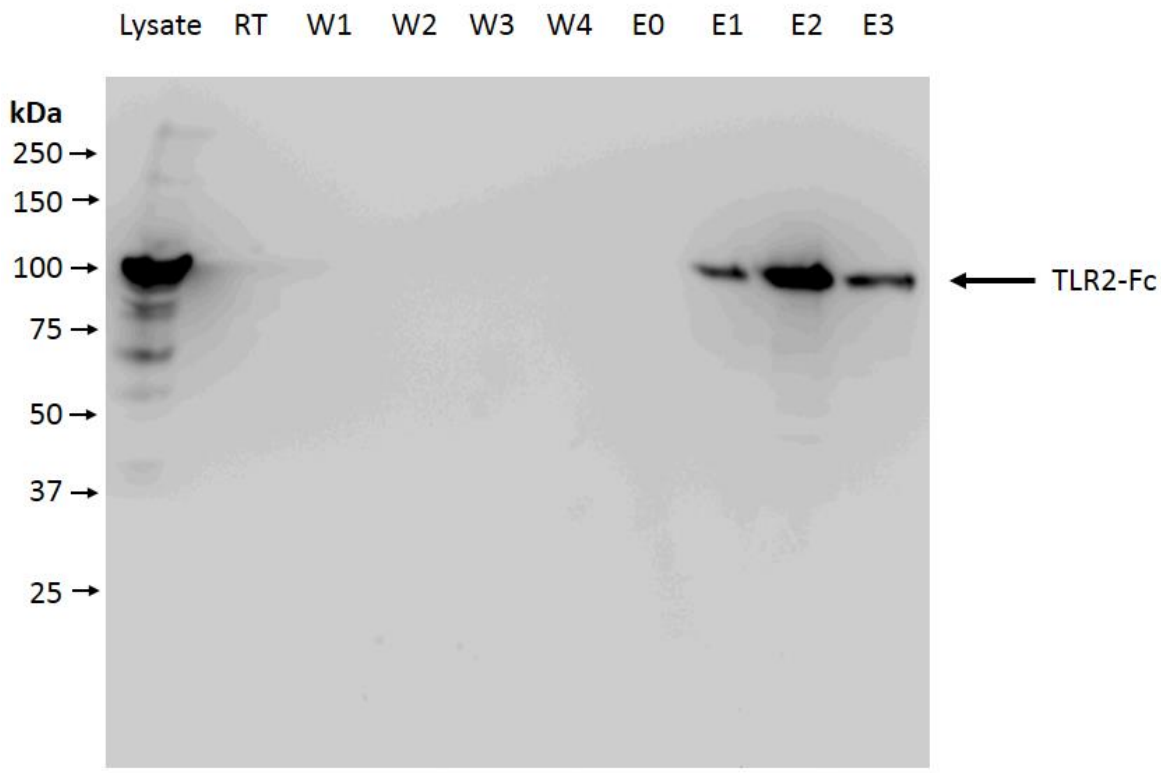

Figure 4.22: Detection of TLR2-Fc in CHO-TLR2-Fc culture supernatants.

Concentrated CHO-TLR2-Fc supernatant was purified using Protein A-agarose columns. Concentrated supernatant was diluted 1:1 with IgG binding buffer and ran through the column, which was washed with binding buffer. Proteins of interested were eluted using IgG elution buffer. The lysate from the TLR2-Fc transfected cells (included as a positive control), column run through (RT), 4 washes (W1-W4), and 4 elution fractions (E0-E3) were blotted for TLR2. The size of the expected TLR2-Fc fusion protein is approximately 95kDa. Note: proteins are not expected in the zeroth elution fraction (E0) since it is collected before the elution buffer can run through the column volume.

4.2.5 *TLR2-Fc depletes IL-10 inducing activity of cell wall preparations*

As a preliminary approach to examine the potential for the TLR2-Fc fusion protein to be used as a tool to identify novel anti-inflammatory ligands, *S. aureus* cell wall digests were pulled down with TLR2-Fc proteins. Purified TLR2-Fc protein preparations were loaded onto Protein A-agarose columns, staphylococcal cell wall preparations were applied to the column, washed, and eluted. These fractions were controlled for concentration by using equivalent volumes for cell wall preparation, run-through collected, and elution. This way, specific cytokine induction capacity could be assess independently of protein concentration. These fractions were used to stimulate human PBMCs for cytokine production.

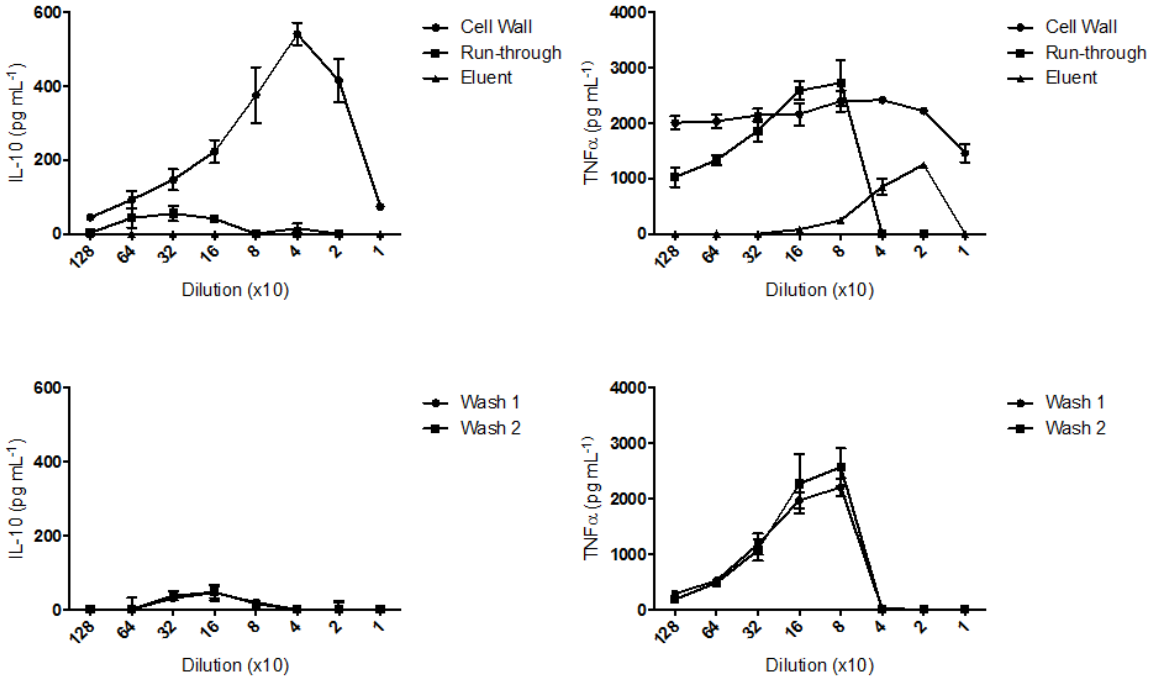

Figure 4.23: Cytokine responses of human PBMCs stimulated with fractions generated by capturing cell wall preparations with the TLR2-Fc fusion protein. Protein A-agarose columns were loaded with TLR2-Fc fusion proteins, and cell wall preparations were run through the column (denoted run-through). The column was washed with IgG binding buffer (Wash 1, Wash 2). The TLR2-Fc and any captured molecules were eluted using IgG elution buffer. The untreated cell wall was also assayed as a positive control of cytokine induction. Each fraction was serially diluted twofold and used to stimulate human PBMCs for cytokine responses.

A strong IL-10 response was observed from the cell wall preparation, but not from the run-through, eluent, or any of the washes (Figure 4.23). The TNF response was strong against the cell wall and run-through, but weaker against the eluent. TNF was also observed in response to the column washes. These results suggest that column capturing using TLR2-Fc is able to deplete the cell wall of IL-10 activity, leaving the TNF response largely intact, since TNF responses were observed in the column run-through. However, it is not known why the column eluent did not induce any IL-10 production.

4.3 *Summary of results*

Here we have shown that cell wall preparations resulting from the enzymatic digestion of *S. aureus* strains can reproduce the IL-10 response induced by heat-killed *S. aureus,* and are additionally able to reproduce the differences between strains of *S. aureus* in their IL-10 inducing capacity. We further demonstrated that through biochemical separation on the basis of apparent molecular weight and electrostatic interactions, staphylococcal cell wall fractions can be enriched for IL-10-inducing capacity. Proteomics analysis on these enriched fractions identified a number of proteins required for microbial adhesion that were anchored to the cell wall through the action of the enzyme Sortase A. Sortase A deficient strains were found to be deficient in IL-10 induction, identifying Sortase A and Sortase A-dependent proteins as possibilities for anti-inflammatory TLR2 ligands.

In an effort to develop novel tools to study the interactions between TLR2 receptor complexes and their ligands, we developed fusion proteins that combined the extracellular domains of TLRs 1, 2, 6, and 10 with the Fc domain of a human IgG1. In a

preliminary study, column capture with TLR2-Fc proteins were shown to deplete cell walls of IL-10 activity, suggesting that the proteins are functional.

5 Chapter 5 – Discussion

Acting as both a clinical relevant human pathogen as well as a common human commensal [8, 60], the pathobiont *Staphylococcus aureus* is an interesting model microorganism to study host-pathogen interactions. Infection with *S. aureus* can lead to life-threatening conditions such as toxic shock syndrome and sepsis, yet it is also carried chronically in the noses of a quarter of the population [8, 60]. One potential explanation for this dual relationship between microbe and host is specific receptor-mediated down-regulation of inflammatory processes at sites of colonization [16, 17], and receptor-mediated modulation of host immunity by microbes has previously been reported [78]. Pattern-recognition receptors such as TLRs are important mediators of inflammation, since they are the earliest sensors of microbial presence during the course of infection [77]. These receptors are known to initiate inflammation upon recognition of foreign microbes through the secretion of cytokines and chemokines [8]. However, TLR activation has also been recently recognized to induce the secretion of anti-inflammatory cytokines such as IL-10 [16-18, 124], an important cytokine for immune regulation given its ability to suppress cytokine production, antigen-presentation, and expression of co-stimulatory molecules.

Activation of TLR4 using LPS has been shown to induce IL-10 secretion [146], but at the time this was most parsimoniously explained as a homeostatic response to curb excessive inflammation, instead of a specifically-activated response. Conceptually speaking, by approaching the problem from the Darwinian context of hosts and parasite coevolution, it

is not difficult to rationalize the possibility of microbes developing strategies to specifically modulate the host inflammatory response [147, 148]. In order to show that such strategies are being utilized, the microbe-activated immunomodulatory response must be distinguishable from host immune homeostatic responses by uncoupling at the level of microbial ligands and/or at the level of host signaling pathways. Recently, activation of TLR2 using staphylococcal peptidoglycan preparations has been also shown to induce IL-10. More importantly, *S. aureus* community strains have been shown to elicit IL-10 production in a manner which does not correlate with their induction of TNF [18]. This suggests that the pro- and anti-inflammatory responses to *S. aureus* can be uncoupled. Previous studies in the Madrenas laboratory have shown that the intracellular pathways which lead to the secretion of pro- and anti-inflammatory cytokines can be uncoupled through their differential requirements for signaling molecules, cytoskeletal reorganization, and phagosome maturation. The results presented in this thesis further this stream of investigation to show that components of the staphylococcal cell wall can be separated to preferentially induce either response, indicating that the pro- and anti-inflammatory responses are activated by distinct sets of ligands.

5.1 *Characterization of S. aureus cell wall extracts prepared by enzymatic digestion*

In order to directly examine components of the staphylococcal cell wall, a protocol to reliably isolate cell wall components needed to be established. Traditional methods of extracting bacterial cell walls, such as treatments with organic solvents, can be harsh and introduce cytoplasmic contaminants into the cell wall preparation [149]. Enzymatic digestion with lysozyme and mutanolysin was chosen since it is a gentle, targeted method

to liberate cell wall-embedded molecules by cutting the linkages between the individual carbohydrate subunits of the peptidoglycan matrix [129]. Since the IL-10 response observed when PBMCs are treated with heat-killed *S. aureus* is reproduced after treatment with staphylococcal peptidoglycan preparations, the cell wall extract would also be predicted to induce IL-10. Additionally, if the TLR2 ligand driving the differential IL-10 responses between isolates is embedded in the cell wall, the differences between the strains when used to stimulate human PBMCs as whole bacteria should be retained when examining their cell walls. Cell walls prepared by enzymatic digestion allowed us to address these questions.

Cell wall preparations were generated using two community isolates of *S. aureus*, a low and high IL-10-inducing strain. S3 consistently induced low levels of IL-10, while S8 consistently induced high IL-10 secretion [18]. When the cell wall and protoplast preparations were tested on human PBMCs for cytokine production, both the IL-10 and TNF responses were strongly activated by the cell wall, but not the protoplast (comprising the cytoplasm and plasma membrane). While the staphylococcal cell wall was able to induce the secretion of both IL-10 and TNF, it was unexpected that the protoplast induced neither cytokine, since components of the bacterial cytoplasm such as nucleic acids are expected to activate other intracellular TLRs to induce pro-inflammatory cytokine release [77]. One explanation may be that after being stripped of the cell wall, the cell membrane of the bacteria could not be efficiently phagocytosed and therefore the other microbial ligands expected to induce a pro-inflammatory response were not available. This explanation is consistent with previous studies showing that TLR

activation enhances the phagocytic function of bone marrow-derived macrophages [150]. Also, through viability staining and flow cytometry, it was found that both the cell wall and protoplast induced substantial cell death in $CD14^+$ monocytes at the 100 and 500 µg/mL concentration (data not shown). This may explain why a small cytokine response is observed following protoplast treatment at small concentrations. Regardless, even if the data points at the two highest treatment concentrations are not interpretable, it is clear that the IL-10 response is activated by the cell wall, not the protoplast, and that the differences between S3 and S8 are recapitulated by their cell wall extracts.

In the interest of purifying the anti-inflammatory ligand(s) from a complex mixture of biological molecules present in the cell wall, we next addressed the question of whether the TLR2 ligand requires protein integrity for signaling. In order to do this, cell walls from the S8 isolate were treated with proteinase K for four hours and inhibited by PMSF. The cell walls treated with proteinase K for four hours were found to induce very low levels of IL-10 in comparison to the zero hour treatment control. Problematically, the zero hour treatment control was itself lower than the cell wall that was untreated. This may reflect the leakiness of the PMSF inhibitor on proteinase K, due to its instability in aqueous solutions. Nonetheless, the difference between the four hour and zero hour treatment conditions is sufficient to show that the cell wall component that induces IL-10 is sensitive to protease treatment. The levels of TNF were more varied between the donors, but overall there was no difference between the 4-hour treated and zero hour treated controls.

Proteins can be a wide range of sizes ranging from small peptides to large macromolecular complexes. The size of the protein is a relevant question in the context of the techniques which can be used downstream to further purify staphylococcal cell walls. Sequential filtration using centrifuge filters generated five size range fractions which were tested in human PBMCs for cytokine secretion. The < 10 kDa and 10-30 kDa fractions were found to induce very low levels of both IL-10 and TNF, comparable to background. The 30-50 kDa fraction induced the secretion of both cytokines, but only at the highest concentration assayed. This lack of dose-dependent response suggests that the ligand itself is unlikely to be found in this molecular weight range. The 50-100 kDa fraction induced high levels of IL-10, and relatively modest levels of TNF, indicating that this molecular weight range may contain ligands which preferentially induced IL-10. The > 100 kDa fraction induced high levels of both cytokines. The anti-inflammatory ligand(s) may be >100 kDa, but are in the same fraction as other pro-inflammatory ligands. The results of molecular weight fractionation using centrifuge filters was sufficient to conclude that anti-inflammatory IL-10 ligands are larger than 30 kDa, and are likely larger than 50 kDa.

5.2 *Fractionation of cell wall preparations by size exclusion chromatography and anion-exchange chromatography*

Size-exclusion chromatography was used to separate the components of the cell wall with much greater resolution. When used to treat human PBMCs for cytokine production, the eluted fractions showed a spread of IL-10 activity across the different fractions, with fractions 7-19 clearly inducing IL-10 secretion above baseline (Figure 4.4). The wide

distribution of fractions that induced an IL-10 response may suggest that unlike the conventional single ligand to single receptor model, there may be a set of ligands which can induce the IL-10 response. However, given the complexity of proteins isolated from the cell wall and relatively low resolution of size-exclusion chromatography, it is also likely that the IL-10 inducing ligands are spread throughout multiple fractions.

In order to test whether the size-exclusion fractions preferentially induced IL-10 over TNF, fractions 10-15 which were observed to induce high levels of IL-10 relative to TNF were concentrated to generate dose-response curves. As a comparison, staphylococcal PGN was used to general control curves for both the anti- and pro-inflammatory responses. The concentrated fractions were found to have a stronger IL-10 response compared to PGN (Figure 4.5), and a weaker TNF response (Figure 4.6), supporting the hypothesis that certain ligands in the *S. aureus* cell wall may preferentially induce the anti-inflammatory response.

Size-exclusion chromatography presented several problems. Size-exclusion chromatography is based on the retardation of smaller molecules by interacting with the porous resin as the sample flows through the column [151]. In size-exclusion chromatography, the samples applied to the column do not actually bind to the column, and thus begin to elute immediately after injection. This is problematic since it presents a maximum possible loading volume of 10 mL for the 220mL column. Increasing the volume loaded and increasing the ratio of loaded volume to the column volume simply further degrades the resolution possible. Another possibility of loading more total

material in the column is increasing the concentration of the cell wall preparation. However, it was difficult to generate cell wall preparations which were sufficiently concentrated to load enough material onto the column to recover sufficient quantifies for the *in vitro* assays. Several potential solutions were explored, including precipitation using trichloroacetic acid (TCA) and ammonium sulfate. Precipitation using 10% TCA was attempted, but the resulting precipitate was difficult to solubilize in aqueous solutions. The TCA precipitates were able to be solubilized in harshly denaturing chaotropic buffers such as 6M guanidinium chloride, but the cell wall preparations lost their ability to induce cytokines following such treatments. Precipitation using ammonium sulfate had a similar problem, with poor yields and low cytokine-inducing activity.

Anion-exchange chromatography was chosen as an alternative method to fractionate the cell wall preparations. Because there is a separate loading and elution phase, the volume that can be loaded was not restricted, allowing separation of cell wall components in their native state with high concentration eluents.

The initial run of anion-exchange with Q-Sepharose resin yielded fractions that were similar to the size-exclusion chromatography in that the IL-10 inducing activity was distributed across many fractions. However, the results were normalized to show the IL-10 levels relative to the volume of the fractions used for each dilution (Figure 4.8). This normalization, where the raw cytokine concentration was divided by the volume of fraction used to make each dilution, was done in order to disregard the concentrations of

proteins which do not induce cytokines. For example, if IL-10-inducing molecules had similar electrostatic interactions with the column resin as another immunologically inert but highly abundant molecule, it would co-elute in a fraction with a high protein concentration. Once diluted based on the protein measurement, the IL-10 response would presumably be low, but this confounding factor would be addressed by normalizing the response to the volume of the fraction used for that dilution. Once normalized, fractions 4 and 5 stood out as eliciting particularly high levels of IL-10 (Figure 4.8). The absence of IL-10 induction in the run-through indicates that most of the IL-10 inducing ligands bound to the column, and suggests that IL-10 inducing ligands are negative charged, binding to the positively charged ammonium ion-exchanger.

These early results were encouraging, but the Q-Sepharose fractions are still complex mixtures of molecules with many components (Figure 4.15). A second run of two-step anion exchange was undertaken, reproducing the first Q-Sepharose separation, and following up with another, higher resolution Mono-Q separation. Fractions which were observed to produce high IL-10 in the first step would be used as the starting material for the second separation, where it could be further resolved by separating into more fractions over a shallower range of salt concentrations. The second run of anion-exchange with the Q-Sepharose resin gave markedly different results from the first. Whereas fractions 4 and 5 gave high levels of IL-10 on the first run, it was fractions 6-8 of the second run which stimulated high levels of IL-10, especially relative to TNF (Figure 4.11). These inter-batch differences highlights the complexities involved in working with biological systems like bacterial cell walls. Looking at IL-10 cytokine concentrations

directly, many fractions were able to induce IL-10, though it was clear only fractions 6-8 induced a significant enhancement of the IL-10/TNF ratio (Figure 4.11). Fractions 3-8 were pooled to maximize the yield of anti-inflammatory and fractionated again on the Mono-Q high resolution resin. None of the MonoQ fractions substantially increased the ratio of IL-10 to TNF induced.

Anion-exchange using Q-Sepharose was done a third time, with results which again differed (data not shown). On this run, the IL-10 inducing ligands ran through the column, and while the run-through robustly induced IL-10, none of the fractions did so. These experiments showed that the physical properties staphylococcal cell walls varied despite efforts being taken to standardize conditions for storage, growth, and enzymatic digestion. This is consistent with published findings which show that cell wall composition is dynamically varied in response to environmental factors [152]. Though useful for enrichment of IL-10-inducing ligands, these chromatographic techniques were ultimate not viable methods to identify staphylococcal anti-inflammatory ligands.

5.3 *Requirement of Sortase A for the IL-10 response and screening of Sortase A dependent strains*

Since a significant enrichment of the IL-10/TNF ratio was observed in the second Q-Sepharose anion-exchange run, fractions 6-8 were sent for mass spectrometry. This identified several proteins which are involved in *S. aureus* virulence in a variety of disease states [46, 140, 141]. These proteins were of particular interest since they belonged to a group of bacterial surface proteins known as microbial surface components

recognizing adhesive matrix molecules (MSCRAMMs) [140]. These MSCRAMMs bind to components of the host extracellular matrix such as fibrinogen, fibronectin, and collagen. The members of the MSCRAMM family share the structural distinction of two adjacent IgG-like folds in the N-terminal region [153, 154]. These proteins are well-conserved, and are also found *S. epidermidis [155]*, *S. pseudintermedius [156]*, Enterococci [157], and Streptococci [158].

MSCRAMMs act as virulence factors to facilitate colonization, enhance tissue invasion, and evade immune responses. *S. aureus* low in fibronectin binding due to mutations in the *fnb* gene were shown to be less virulent in rat models of endocarditis [159, 160]. Deficiencies in *cna*, which encodes for a collagen binding protein, leads to lower rates of arthritis in murine models of septic arthritis [161]. High expression of MSCRAMM genes is correlated with nasal colonization [15], and ClfB is shown to direct contribute to nasal colonization of *S. aureus* in mice [162] and humans [163]. Several MSCRAMMs, including ClfA, SdrE, and Cna, have been shown to help *S. aureus* evade killing by the complement activation [164-166]. Due to the important functions that these MSCRAMMs play in *S. aureus* pathogenesis, as well as the tempting rationale offered by innate receptors that recognize well-conserved virulence factors, we prioritized MSCRAMMs for further investigation. These molecules shared the distinction of requiring Sortase A for anchoring to the cell wall [136].

Sortase A was first discovered in *S. aureus* in 1999 in a forward genetics screen which identified a mutant defective in the anchoring of proteins to the cell wall [137]. Sortase A

was found to catalyze a transpeptidation reaction by recognizing a conserved LPXTG sequence [137]. Sortase A cleaves the protein between the threonine and glycine residues, and transiently binds the threonine end to an active site cysteine before attaching the threonine residue to the lipid II peptidoglycan precursor.

Sortase A catalyzes the anchorage of various staphylococcal virulence factors to the cell wall [137], and several of these proteins were identified in the proteomics analysis of anion-exchange cell wall fractions. We obtained Sortase A deficient *S. aureus* strains and tested these for IL-10 induction. The Sortase A-deficient *S. aureus* induced very little IL-10, while showing an identical TNF induction profile compared to the parental strains (Figure 4.16). These data suggest that the presence of Sortase A is required for IL-10 induction in human PBMCs. Future work to support this conclusion should include complementing the Sortase A gene back into deficient strains to verify recovery of IL-10 induction.

Twenty genes in *S. aureus* were found to carry the LPXTG targeting sequence recognized by Sortase A. Of these, 8 can be found in the Nebraska Transposon Mutant Library, a collection of approximately 2000 strains generated on the same parental *S. aureus* by transposon insertion [138]. This panel of strains were obtained, and the relevant strains grown and tested on human PBMCs for cytokine induction. While the Sortase A-deficient strain in the NTML library reproduced our previous results, the Sortase A target proteins were either unchanged or even enhanced in terms of IL-10-inducing capacity (Figure 4.17).

This unexpected result of enhanced IL-10 induction could be due to the removal of competition from the cell wall space. If a particularly prominent cell wall component is knocked out, it is possible that other proteins, some of which may induce IL-10, gain greater access to the space on the cell wall. The removal of certain cell wall components could also affect the architecture and organization of the cell wall in unpredictable ways. In the future, the other 12 proteins which are targeted by Sortase A should be tested to see if any of them are deficient in IL-10 induction. Although genetic manipulation of *S. aureus* has been historically been difficult due to well-conserved restriction systems, technologies which make site-direct mutagenesis possible have been recently developed [167]. These systems can be used to inactivate Sortase A targets in *S. aureus* and examine their cytokine responses. If none of these targets are deficient in IL-10 induction, it is tempting to speculate that TLR2 may recognize the Sortase A protein itself, especially since Sortase A is well conserved in *S. aureus* and other Gram-positive bacteria. Alternatively, multiple ligands could be synergistically activating the anti-inflammatory response, requiring combinatorial analysis of Sortase A knockouts in order to observe the IL-10-deficient phenotype.

5.4 *Development of TLR-Fc fusion proteins as tools to study novel TLR ligands*

TLR-Fc fusion proteins that combine the extracellular domains of TLR1/2/6/10 to the IgG1 heavy chain Fc domain can be powerful tools to identify TLR2 and TLR2 heterodimer ligands on the basis of their biochemical affinity to receptor complexes. This approach is advantageous over historical methods since it relies neither on an extremely

pure preparation of microbial components, nor reporter genes. The ectodomains for TLRs 1, 2, 6, and 10 were subcloned into the pFuse plasmid, allowing express of the TLR-Fc fusion protein in mammalian systems. This approach has previously been reported, where a TLR2-Fc protein was constructed and utilized in an ELISA-like assay to determine interaction between the TLR2 ectodomain and acute-phase serum amyloid A protein [143]. We aimed to use the extracellular domain of TLR2 receptor complexes to immunoprecipitate ligands from a complex cell wall preparation.

The Lonza nucleofection technology was chosen to transfect CHO cells with the TLR-Fc constructs. This electrophoresis technology was shown to have high efficiency, since CHO cells transfected with the pmaxGFP plasmids expressed GFP 24 hours after transfection in high proportion (> 80%) (Figure 4.19). CHO cells were also transfected with TLR1-Fc, TLR2-Fc, TLR6-Fc, and TLR10-Fc. Cell lines for each of these fusion proteins have been established, but so far only TLR2-Fc has been validated to express the gene of interest through western blotting, both of the CHO cell lysates as well as culture supernatants.

As a preliminary experiment to examine whether the TLR2-Fc was able to bind to components of the cell wall, a column capture experiment was carried out where TLR2-Fc linked to Protein A-agarose was used to capture components of *S. aureus* cell wall preparations. Surprisingly, we found that the TLR2-Fc in the absence of any other heterodimer partners was able to deplete the cell wall preparation of IL-10-inducing capacity, since the cell wall preparation, but not the column run-through, was able to

stimulate IL-10 responses (Figure 4.23). Recently, SitC, a triacylated lipopeptide, has been shown to induce IL-6 and TNF in murine peritoneal macrophages, a process found to be dependent on TLR2 and MyD88, but neither TLR1 nor TLR6 [168]. It is possible that TLR2 homodimers are sufficient for anti-inflammatory TLR2 signaling, but this remains a contentious claim. The evidence against TLR2 homodimerization is an early study where the extracellular domains of TLR2 were replaced with CD4 molecules, and cross-linked using anti-CD4 antibodies [142]. However, it should be noted that this early study used the production of TNF as a readout for TLR signaling.

Also puzzling is the absence of IL-10 induction in the eluent of the bound material. The cell wall was depleted of IL-10-inducing activity, but it is not found in the washes nor the elution (Figure 4.23). A possible explanation for this may be that the TLR2-Fc proteins are bound to the anti-inflammatory TLR2 ligands and compete for binding with the TLR2 molecules on the human PBMCs. This is possible since the protein complexes are eluted with a buffer which is able to free the TLR2-Fc proteins from the agarose-linked Protein A. These immunoprecipitation experiments still require optimization in terms of the buffers and elution conditions used. With optimization, immunoprecipitation experiments using TLR2 with its dimerizing partners may provide novel insights to microbial TLR2 ligands.

5.5 *Conclusions and future directions*

In conclusion, the work presented here show that the pro- and anti-inflammatory responses to *S. aureus* can be uncoupled on the level of cell wall fractions which may

preferentially induce each response. Moreover, the IL-10 response was shown to be dependent on the transpeptidase Sortase A, which anchors proteins carrying the LPXTG sequence to the cell wall. If Sortase A is involved directly in the anchoring of IL-10-inducing ligands, this finding greatly narrows down the possibilities, since Sortase A has 20 known targets. In this case, site-directed mutagenesis on *S. aureus* may be a good option for future investigation into cell wall embedded IL-10-inducing molecules. It is also possible that a deficiency in Sortase A changes the architecture of the cell wall in a way that is not conducive to anti-inflammatory signaling. In this case, returning to biochemical fractionation may be the best way forward. Another intriguing direction is the composition of the bacterial supernatants. Since Sortase A-deficient bacteria would be expected to shed LPXTG-targeted proteins into the supernatant, these supernatants may contain molecules which may be involved in TLR signaling, whether towards pro- or anti-inflammatory responses.

TLR-Fc fusion proteins were developed, and preliminary results indicate exciting possibilities in their use for elucidating novel TLR ligands. The expression of TLR1-Fc, TLR6-Fc, and TLR10-Fc need to be validated biochemically and purified. Conditions for ligand-capture using these TLR-Fc proteins need to be optimized, but preliminary results are encouraging.

These data act as a corollary to our earlier work showing that the intracellular pathways leading to pro- and anti-inflammatory cytokine responses can be uncoupled biochemically [18]. The discovery of distinct sets of ligands which stimulate pro- and

anti-inflammatory cytokine induction preferentially solidifies the conception of TLR-mediated anti-inflammatory responses as being resultant from specifically evolved bacterial ligands signaling on receptor complexes to activate specific intracellular pathways which lead to each type of response. The availability of the TLR-Fc fusion proteins will bring the question of differential signaling from TLR2 receptor complexes to the forefront. Some have claimed that TLR2 heterodimerization contributes only to ligand diversity, but not to differential intracellular signaling [118]. These, and other questions, stand ready to be answered with new perspectives and new tools.

The discovery of molecules which can specifically activate anti-inflammatory responses may inform future immunomodulatory therapeutic strategies. Moreover, an understanding of how *S. aureus* is able to downregulate the host inflammatory processes to maintain its commensal state may shed new light on the spectrum of interactions between human hosts and microbes, which range from aggressive pathogenicity to true mutualism.

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