# Crouching neutrophils, hidden bacteria

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**Crouching neutrophils, hidden bacteria**
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**About the title and cover.** The title of this thesis is inspired by one of my favorite Chinese movies: Crouching tiger, hidden dragon. This movie was released in 2000 and directed by the famous Chinese director Ang Lee. The cover depicts a scene from where the leading characters fight while balancing on bamboo. In this thesis, we focus on the constant tug of war between neutrophils and bacteria. Just like the characters on the bamboo, neutrophils and bacteria have a complicated relationship where fighting, balance, and interaction are extremely important.



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# Crouching neutrophils, hidden bacteria

**Tijgerende neutrofielen, verborgen bacteriën**
(met een samenvatting in het Nederlands)

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CHAPTER 1

# General introduction Neutrophils vs bacteria: a complex molecular battle

Yuxi Zhao

## Abstract

There is an ongoing biological battle between bacteria and the hosts that they colonize and infect. Invading bacteria can be successfully eliminated from the body by professional phagocytes. Neutrophils are the most abundant phagocyte of the innate immune system, and play a crucial role in defense against invading bacteria. Neutrophils sense their environment through expression of a variety of innate immune receptors that detect microbes, microbial products and/or host factors. Upon stimulation, neutrophils engulf and kill bacteria through effector functions such as phagocytosis, degranulation and NETosis. Not surprisingly, pathogenic bacteria have often evolved sophisticated approaches to evade neutrophil attacks. This includes expression of various "immune evasion" molecules. Thus, there is a constant tug of war between neutrophil and bacteria. In order to understand how bacterial pathogens cause disease, we need comprehensive knowledge of when, where and how bacteria evade neutrophil attacks.

In this review, we first describe the role of neutrophils in the fight against the bacterial infection. We then highlight the different bacterial secreted "immune evasion" strategies that enhance resistance to neutrophil attacks. Finally, we emphasize future research perspectives to improve knowledge of neutrophil function & bacterial pathogenesis. This includes an overview of receptors for which the expression or function on neutrophils have not yet been characterized, and introductions to novel high throughput techniques to identify new host-pathogen interactions. Collectively, this information is fundamental to a better understanding of innate immune responses, to more accurate knowledge of bacterial pathogenesis and to the development of novel treatments for infectious and inflammatory diseases.








# Part 1: Neutrophil function

Neutrophils (also known as PMN [polymorphonuclear leukocytes]) are the most abundant phagocytic cell in the human blood, accounting for approximately 60% of all circulating leukocytes (1). Neutrophils are ready-to-go cells and provide the front line of host defense against invading microbes. In addition, they have a pivotal role in orchestrating inflammation (2, 3). Their critical role in bacterial defense is highlighted in patients that have inherited or acquired neutrophil dysfunction, e.g. leukocyte adhesion deficiency (4) and chronic granulomatous disease (5), as well as neutropenic patients after such as bone marrow transplantation or cancer treatments (6, 7), that are associated with an increased risk of severe bacterial infections. The major task of neutrophils is to protect the body from harmful microbial infections, especially those caused by commensal bacteria that are opportunistic pathogens. In order to efficiently eliminate invading bacteria, neutrophils must 1) migrate to the site of infection from the bloodstream, 2) sense the presence of invading microbes, 3) kill bacteria through phagocytosis and generate ROS (also known as the oxidative burst) in the phagosome, through the release of different bactericidal components by degranulation or through the release of DNA and formation of neutrophil extracellular traps (NETs). Importantly, during this process neutrophils must produce balanced inflammatory responses to ensure return to an non inflamed state after the microbial clearance. Many different immune receptors are involved in generating, shaping and modulating neutrophil activity, effector functions and responses to microbes (8, 9). In part 1, we focus on the host perspective and how neutrophils recognize microbes and modulate their response via diverse immune receptors.

## 1.1 Neutrophil biology

Neutrophils are generated in the bone marrow from the myeloid hematopoietic system and mobilized rapidly into blood circulation. They share similar characteristic features with other myeloid cells, such as monocytes/macrophages and mast cells (10). Neutrophils are short-lived cells in contrast to other phagocytes. They survive in the blood shortly and die by a spontaneous apoptotic program (11). Apoptotic neutrophils are subsequently removed by macrophages. Though it remains unclear why neutrophils possess such a short lifespan, it is likely to be related to neutrophil homeostasis, since an excessive neutrophil abundance in the blood results in unnecessary and damaging inflammation (12, 13).

Neutrophils are terminally differentiated cells and they normally do not express any new proteins in the bloodstream. Instead, neutrophils synthesize all the proteins they require whilst they reside in the bone marrow, and store many of

these proteins in different granule populations (10). Neutrophil granules have been subdivided into four different classes based on their resident cargo molecules: azurophilic (or primary) granules, specific (or secondary) granules, gelatinase (or tertiary) granules, and secretory vesicles (SVs). The primary granules contain a high amount of myeloperoxidase (MPO), the secondary granules neutrophil serine proteases (NSPs), the tertiary granules lactoferrin and gelatinase, and the SVs mainly contain cell surface receptors (14, 15). Additionally, SVs and granules contain immune receptors that are embedded in membranes. All the stored proteins and receptors are important for the antimicrobial and proinflammatory missions of the neutrophils (14). Once released into the circulation, neutrophils are primed and activated by different signals of infection and inflammation. Upon receiving these signals, neutrophils mobilize their SVs and/or granules which move to and fuse with the cell membrane. The surface content of neutrophils changes this process, as membrane anchored receptors in SVs and granules are now located at the neutrophil surface. For example, the expression of surface receptor CD11b will be increased and the surface expression of surface receptor CD62L will be decreased. Another example is TREM-like transcripts (TLT)-2 (TLT-2), TLT-2 plays an important role in potentiating neutrophil antibacterial activity and chemotaxis, resides in the primary (azurophilic) granules and is up-regulated through degranulation after stimulation with inflammatory mediator (16, 17)**.** Thus, the resting and the primed neutrophils differ in surface receptor content, and sense and respond to their environments differently. In addition, the soluble proteins from SVs and granules are released in to the local environment. For example, when neutrophils get activated by either bacterial proteins or signals from host cells, they release MPO from their primary granules. Collectively, these process help to ensure that neutrophils initiate the designated antimicrobial killing programs at the local infection site. This includes recognition, phagocytosis and killing of the infection particle (14, 18, 19).

## 1.2 Migration to the site of infection from the bloodstream

When bacteria invade their host they initiate a cascade of events that signal neutrophils to migrate from the bloodstream towards the site of infection (Fig 1). Neutrophils first need to cross the vascular endothelium, a process known as extravasation. Extravasation encompasses four important steps; rolling, adhesion, crawling and transmigration. Each step is regulated by cross talk between neutrophil receptors and endothelial cells. Initial attachment of neutrophils to endothelial cells is determined by the endothelial cells, which react to stimuli such as TNFα, IL-1β, and IL-17 that are generated during infection or inflammation. Such stimulation results in upregulated expression of P-selectin and E-selectin, as well as members of the integrin superfamily (the ICAMs and VCAMs, detailed later)







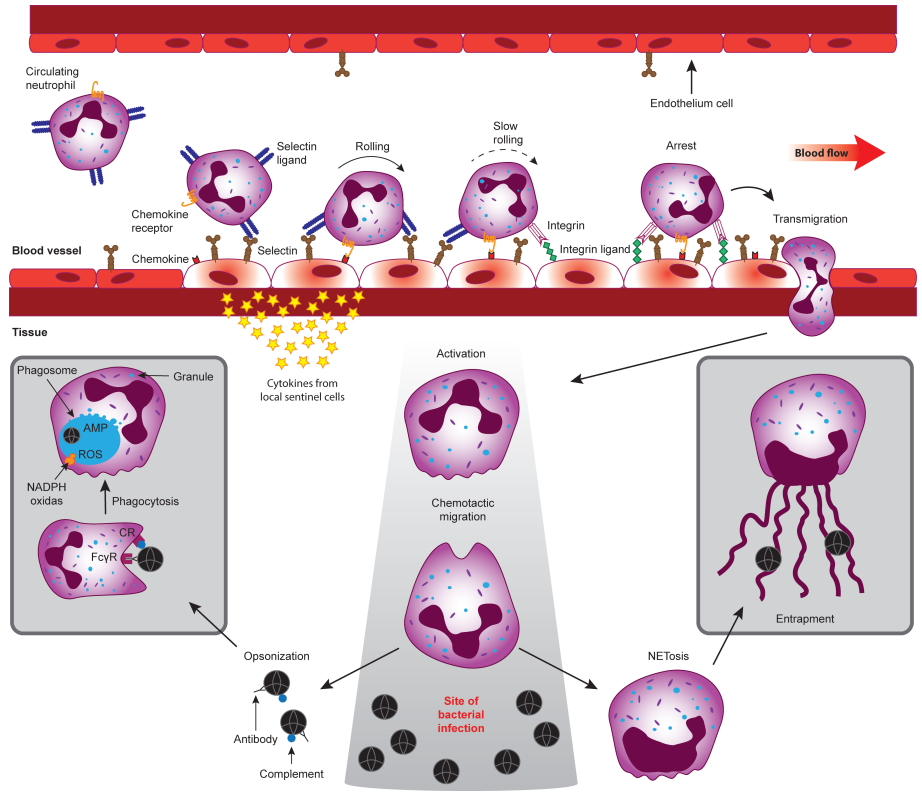


**Figure 1. Neutrophil extravasation to the site of infection and killing mechanisms.**

Cytokines from local sentinel cells activated endothelium cells. Recruitment of neutrophils begins and followed by rolling on the activated endothelium. Upon stimulation, Neutrophils captured through the interaction of selectins on endothelial cells with their ligands on neutrophils. Selectins and chemokines induce integrin-mediated slow rolling and neutrophil arrest. Neutrophils undergo integrin-mediated crawling to a site for transmigration. This step is followed by neutrophil transmigration through the endothelium into the tissue, a process known as extravasation. In tissue, neutrophils are directed by a chemotactic gradient toward site of infection, followed by recognition and subsequent phagocytosis. Phagocytosis of bacteria is dependent on the deposition of complement and immunoglobulins, which are recognized by complement receptors (CRs) and Fcγreceptors (FcγRs). Inside the neutrophil phagosome, bacteria are killed by antimicrobial proteins (AMPs) released upon granule fusion and by NADPH oxidase-derived reactive oxygen species (ROS). Alternatively, neutrophils kill bacteria by release of NETs.

on the luminal surface (20–22). This provides an environment for neutrophils to adhere to endothelial cells through the receptor P-selectin glycoprotein ligand-1 (PSGL-1). These interactions ensure that free-flowing neutrophils start to slowly roll along the endothelial cell wall (23). After rolling is initiated, neutrophils fully adhere to endothelial cells through high-affinity interactions of their surface adherence receptors, LFA-1 and Mac-1, with the endothelial intracellular adhesion

molecule 1 (ICAM-1). This subsequently allows neutrophils to begin crawling on the endothelium surface (24–26). After identification of a suitable transmigration site, neutrophils transmigrate through the endothelial junction mediated by numerous activated endothelial cell junctional molecules, e.g. PECAM-1, CD99, ICAM-2, ESAM, and members of the junctional adhesion molecule (JAM) family (26–28).

Next, neutrophils are directed to the local site of infection through the sensing of chemoattractant gradients, which can be derived from either bacterial secreted molecules or host factors, such as chemokines that are secreted by activated cells (29) and complement factors formed during complement activation (30). This process is known as chemotaxis (31). In particular, neutrophils express a diverse set of immune receptors, mostly belonging to G protein-couple receptors (GPCRs), that sense these environmental stimuli. Bacterial secreted molecules provide a critical trigger of chemotaxis, in particular N-formylated peptides (e.g. formyl-methionyl-leucyl-phenylalanine, fMLP) (32, 33). For example, fMLP binds formyl peptide receptor 1(FPR1), inducing the neutrophil to migrate up the fMLP gradient. Chemokines such as IL-8 binds to CXCR1/2, thereby also directing neutrophil to the site of infection (34). Products of the activated complement system like the anaphylatoxins C3a and C5a, bind to C3aR and C5aR respectively, and both act as chemoattractants by attracting neutrophil to the site of infection. Collectively, gradients of microbial products and host factors provide mechanisms to guide neutrophils to migrate to local sites of infection.

### 1.3 Priming and activation of the neutrophil

At the local infection site, neutrophils are primed and activated by a diverse range of microbial products and inflammatory stimulants. Examples of well-known priming and activation molecules of neutrophil are: the complement components C3a and C5a (35), interleukin-8 (IL-8) (36), granulocyte colony-stimulating factor (G-CSF) (36), tumor-necrosis factor (TNF-α) (37), interferon-γ (IFN-γ) (38), bacterial lipoproteins , bacterial DNA (39), and bacterial formylated peptides (32). Some of the functional changes that can be observed in primed neutrophils include the following: altered composition of surface receptors and adhesion molecules and enhances the neutrophil's functional response, e.g. adhesion, phagocytosis, degranulation, and superoxide production (ROS). An example of this phenomenon is that resting neutrophils express low affinity Fcγ receptors, but activated neutrophils express high affinity Fcγ receptors. Furthermore, this enhances Fcγ mediated neutrophil degranulation and ROS production (40, 41).







### 1.4 Phagocytosis of bacteria to the phagosome

Phagocytosis, or "cell eating", is defined as the engulfing of other cells, cell fragments and microorganisms into the phagosome. Phagocytosis can be divided into three steps: 1) attachment to the opsonized particle recognized via specific receptors, 2) ingestion of the attached particle into an intracellular phagosome by invagination of the cell membrane, and 3) intracellular killing of the particle in the fused phagosome and lysosome, known as the phagolysosome (42). Therefore, recognition of foreign particles via immune receptors plays an important role in initiation of phagocytosis. Foreign particles, such as bacteria, fungi, and parasites display many pathogen-associated molecular patterns (PAMPs) that can be detected by several pathogen recognition receptors (PRRs), such as, Toll-like receptors (TLRs) (43), mannose receptor (CD206) (44) and Dectin-1 (CLEC7A) (45). Optimal phagocytosis also requires opsonization, a process in which the bacterial surface is decorated with molecules, called opsonins, that are recognized by specific neutrophil surface receptors. Complement components and immunoglobulins are the two most abundant and efficient opsonins in serum. They are deposited on the surface of bacteria and recognized by complement receptors (CRs) and Fc receptors (FcRs) respectively. More specifically, CR1 and CR3 recognize C3b (and iC3b) mediated opsonization, FcγRIIa and FcγRIIIb recognize IgG mediated opsonization, FcαRI recognizes IgA mediated opsonization. Moreover, the combination of complement and IgG opsonization, triggers more efficient neutrophils phagocytosis (46). More detailed description of neutrophil mediated phagocytosis of bacteria can be found in excellent reviews (1, 42, 47).

Inside the neutrophil phagosome, bacteria are killed by an arsenal of anti-bacterial proteins stored in neutrophil granules and by membrane-associated NADPH-oxidase that induce a massive generation of ROS, a process known as oxidative burst. ROS represents a complex and dynamic mixture of highly reactive molecules that differ in their stability in, reactivity to, and permeability through membranes, such as superoxide radicals, hydrogen peroxide, hypochlorous acid, hydroxyl radicals, and chloramines. In resting neutrophils, subunits of the NADPH oxidase complex are separated in cytosol and membrane compartments. In activated neutrophils, the cytosolic components translocate to the plasma and/or phagosome membrane and associate with membrane compartments to form the active oxidase (48). The oxidase transfers electrons from cytosolic NADPH to intraphagosomal molecular oxygen to produce superoxide (49). The NADPH oxidase is essential to eliminate bacterial and fungal pathogens, as inherited defects in components of this enzyme system predispose individuals to severe and/or fatal infections (50).

### 1.5 Degranulation & NETosis

Neutrophils are terminally differentiated cells that carry a large number of antimicrobial peptides and proteases in their granules. In a process called neutrophil degranulation, granules translocate to the phagosomal or plasma membrane, where they dock and fuse with the membrane to release their contents (51). The content of these granules is diverse, but many different molecules, such as MPO, bactericidal/permeability-increasing (BPI) protein, lysozyme, defensins, NSPs (neutrophil elastase, proteinase 3, and cathepsin G), lactoferrin, NGAL (neutrophil gelatinase associated lipocalin), hCAP-18, and gelatinase (15, 52), all have major functions in directly or indirectly killing of microbes.

Neutrophils are also able to kill bacteria by releasing DNA content and granules and inducing neutrophil extracellular traps (NETs), which trap and thereby prevent further dissemination of the pathogens (53). NETs are formed via a novel type of cell death called NETosis, which requires the formation of ROS. Several different agonists trigger NET formation, including cytokines, microbial components, and bacteria itself (54, 55). *Yipp et al*. shown that *in vivo* NETosis is tightly regulated through TLR2 and complement-mediated opsonization (56). Moreover, studies have shown that in the bloodstream, when antimicrobial peptides (AMPs) associate with NETs, AMPs can reach their antimicrobial concentrations to be effective in killing microbes (53). More detailed mechanisms of neutrophil intracellular and extracellular killing are present in these excellent papers (1, 2, 46, 51, 52)

### 1.6 The importance of neutrophils suppression

Neutrophil effector functions are highly efficient at eliminating invading microbes. However, if these mechanisms become activated at the wrong time and/or in the wrong place this can lead to host damage. Therefore, activatory and inhibitory receptors provide important mechanisms that modify activation thresholds and ensure the generation of appropriate immune responses. These receptors are illustrated in Fig 2. In particular, neutrophils express an array of inhibitory receptors that increase the activation threshold (8). They can suppress cell activation through opposing signaling mechanisms and thereby ensure a balanced neutrophil response upon the appropriate amount of stimulation. Some of those inhibitory receptor are well characterized, such as signal regulatory protein α (SIRPα), a myeloid inhibitory receptor and expressed on the human neutrophil, containing three Ig-like domains and a cytoplasmic region containing two ITIMs. The principal ligand for SIRPα is CD47, an ubiquitously expressed glycosylated protein (57, 58). CD47 functions as a "don't eat me signal" by binding to inhibitory SIRPα and thereby inhibits certain effector functions of neutrophils. LAIR-1 (leukocyte-associated immunoglobulin-like receptor-1) expressed on activated neutrophils and binds to collagens. LAIR-1 has been shown to inhibit cytotoxicity, degranulation or calcium mobilization in different immune cells, but has not been directly shown for neutrophils yet (59, 60). However, for other inhibitory receptors expressed on neutrophils that there is no or limited knowledge of their functions and ligands. This includes receptors of the leukocyte immunoglobulin-like receptors (LILR) family that are immunomodulatory receptors expressed on a variety of immune cells (60, 61). LILR can divided into activatory (LILRA) and inhibitory (LILRB) by containing two different cytoplasmic region (8). Consequently, binding of ligands to LILRA or LILRB is hypothesized to lower or enhance the immune activation threshold. Studies have shown that some LILRs can modulate neutrophil activation (62, 63), while, robust knowledge of expression and functions of all LILRs on neutrophils is lacking.

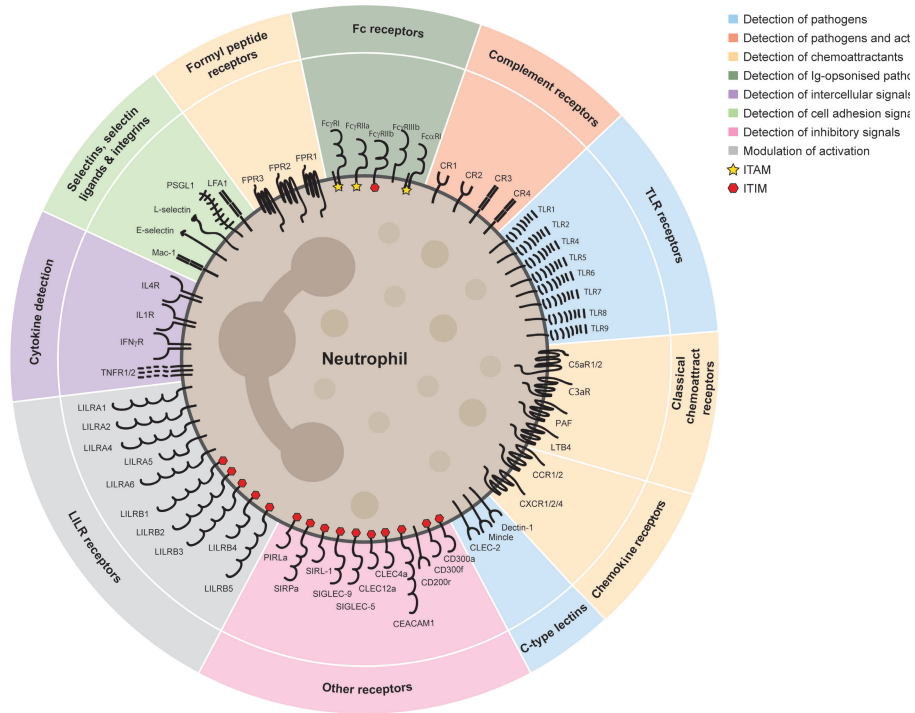


**Figure 2. Neutrophil receptors.**

## Part 2: Bacterial neutrophil evasion

The human body is a constantly changing ecosystem comprised of trillions of microbial organisms collectively known as the microbiome (64). Most bacteria in the human microbiome exist in the gut flora and on the skin, and reside harmlessly by the protective effects of the immune system. These bacteria are therefore called 'commensal bacteria'. However, several 'commensal bacteria' are opportunistic pathogens and cause both mild and/or severe infectious diseases. These bacteria have co-evolved with their hosts, and have evolved mechanisms to evade immune responses, survive within their host and exert pathologic effects. For instance, *Staphylococcus aureus* (*S. aureus*) is a commensal of the skin and anterior nares, but is able to cause a range of minor skin and soft tissue infection to more severe life-threatening systemic infection (65).

In order for bacteria to become a pathogen, they must be able to adhere to, grow on, and invade the host. It is evident that pathogenic bacteria have evolved highly sophisticated "immune evasion" mechanisms to evade neutrophil attacks. Indeed, the ability of bacterial pathogens to avoid killing by neutrophils often involves multiple strategies or characteristics. Many pathogenic bacteria express capsules to mask surface antigens and reduce opsonisation and immune recognition. Furthermore, many pathogenic bacteria express surface proteins and/or secreted molecules that possess immune evasion properties. These "immune evasion" molecules are diverse in composition and function, and collectively have the potential to alter or inhibit the ability of neutrophils to migrate to local sites of infection migration, to recognize and phagocytose microbes and to effectively kill bacteria. One of the most extensively studied organism that deploys a large variety of different immune evasion strategies is *S. aureus*. This bacterium secretes dozens of immune evasion molecules that each target and inhibit or modify a different neutrophil function (47, 66). In part 2, we use *S. aureus* as an example to explain the different strategies utilized by bacteria to subvert neutrophil recruitment and functions (Fig 3). Following the host response sequence as described in part 1, the bacterial molecules that counteract the neutrophil extravasation, migration, phagocytosis and killing will be described.

### 2.1 Evading neutrophil extravasation and migration

*S. aureus* secretes a number of molecules that inhibit neutrophil chemotaxis recruitment. In general, these molecules can provides three different strategies to control neutrophil migration. Firstly, bacteria are able to interrupt the recruitment of neutrophils to the activated endothelium. For example, *S. aureus* molecules Staphylococcal superantigen-like protein 5 (SSL5) and SEIX that both bind to

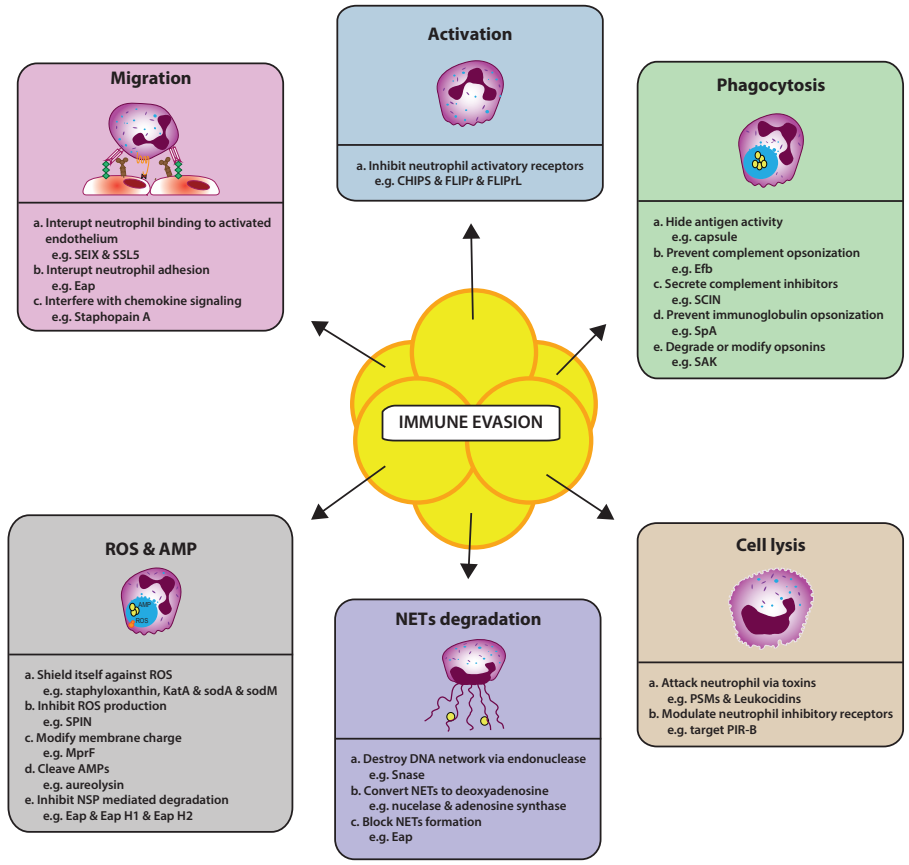


**Figure 3. Evasion of neutrophil killing by *S. aureus* strategies overview.**

PSGL-1. SSL5 prevents PSGL-1 interactions with P-selectin in a sulfation and sialylation dependent manner (67, 68), whilst SEIX interacts with PSGL-1 in a glycosylation dependent manner (69). Secondly, bacteria can release molecules that bind to neutrophil receptors involved in adhesion, thereby reducing adhesion of neutrophils to endothelial surfaces. For example, *S. aureus* extracellular adherence protein (Eap) directly binds ICAM-1 to prevent neutrophil adherence to endothelial cells (70). Thirdly, bacteria can prevent neutrophil transmigration though the endothelial junctions. So far, there is no evidence that shows that *S. aureus* can directly inhibit neutrophil transmigration through the endothelial junction. However, *S. aureus* secretes proteases and several other molecules that target neutrophil chemoattractants and chemokine receptors thereby inhibiting neutrophil migration. This includes a proteinase named Staphopain A, that is

secreted by *S. aureus*, which interferes with chemokine signaling. It acts by cleaving the N-terminus of CXCR2, and thereby hampers neutrophil migration toward CXCR2 chemokines (71). Another well-characterized molecule SSL5, which inhibits C3a and C5a induced activation by binding glycosylated N-termini of the C3aR and C5aR GPCRs (72). Similarly, SSL10 inhibits neutrophil migration by binding and inhibiting the functions of CXCR4 (73). Other examples include chemotaxis inhibitory protein of Staphylococcus (CHIPS), a small protein of 14.1 kDa, that inhibits neutrophil migration towards chemoattractants of C5a and fMLP by binding and inhibiting C5aR and FPR1 receptors, respectively (74, 75). Similar to CHIPS, FPR2 inhibitory protein FLIPr and its homologue protein FLIPr-like both inhibit FPR1 and FPR2 that recognize fMLP chemoattractants (76, 77).

### 2.2 Evading neutrophil phagocytosis

The most efficient means for a bacterium to prevent phagocytosis is to intervene with opsonization. This can be achieved by either directly hiding or masking of surface antigens by production of a polysaccharide capsule, or indirectly by exploiting bait molecules to inactive opsonins. Many human pathogenic bacteria are known to produce a capsule including *S. aureus*, Group A *Streptococcus*, and *Streptococcus pneumonia*. In *S. aureus*, around 70% of clinical strains express one of two different capsular forms, capsular polysaccharide 5 (CP5) or capsular polysaccharide 8 (CP8). These capsules allow *S. aureus* to hide their antigenic or immunogenic proteins to resist opsonophagocytosis (78). Many other strategies are utilized by *S. aureus* to evade complemented- and antibody-mediated phagocytosis. For example, Staphylococcal extracellular fibrinogen-binding protein (Efb) binds fibrinogen in order to cover *S. aureus* by a thick layer of fibrinogen. This fibrinogen layer acts like a pseudo capsule and inhibits phagocytosis by preventing the phagocytic receptors of recognizing the opsonins C3b and IgG (79). In addition to anti complement-opsonic strategies, *S. aureus* also secretes many molecules that act as complement inhibitors. For example staphylococcal complement inhibitor (SCIN) binds C3 convertase and thereby prevents massive C3b deposition (80, 81), while SSL7 binds fluid phase C5 and inhibits C5a generation (82, 83) . All of these *S. aureus* molecules generally affect the conversion of complement through binding major complement convertase components, and therefore hamper and hinder the complement cascade at several different stages. There are also several other *S. aureus* complement inhibitors, such as Ecb and Sbi, that are reviewed elsewhere (84–87).

Another anti-opsonic strategies also used by *S. aureus*, employs immunoglobulin-binding proteins. Staphylococcal protein-A (SpA) is the first immune evasion protein described to have anti-opsonic properties. SpA is a cell wall-anchored protein, that is also released into the supernatant. SpA contains four or five homologous domains







with strong binding affinity for the Fc region of human IgG (88). SpA also binds to the Fab fragment of Ig in a variable heavy 3(VH3) dependent manner (89, 90). Additionally, *S. aureus* secretes a second immunoglobulin-binding protein (Sbi) (91). Sbi is a multifunctional protein, which not only exhibits comparable immunoglobulin binding affinity to SpA (92), but also acts as a complement inhibitor and interferes with innate immunity recognition (93). Furthermore, another protein with dualistic functions is SSL7, that besides it ability to bind C5 binding, also binds IgA and thereby has a role in inhibiting opsonophagocytosis by interfering with FcαR activation (82). In addition, SSL10 binds specifically to human IgG1 with consequences for FcγR recognition and complement activation (94). To evade opsonophagocytosis, *S. aureus* also secretes immune evasion molecules targeting the phagocyte receptors for these opsonins. FLIPr, and FLIPr-like, besides their function as FPR inhibitors mention above also act as potent FcγR antagonist. They bind FcγR and abrogate FcγR-mediated effector functions, including opsonophagocytosis and subsequent intracellular killing of *S. aureus* by neutrophils (95).

Bacteria may also decrease neutrophil opsonophagocytosis by degrading or modifying opsonins. For example, *S. aureus* also secrets a protein named Staphylokinase (SAK), which can form a complex with human plasminogen and convert plasminogen into active plasmin, a broad-spectrum proteolytic enzyme. Plasmin is shown to cleave bacterial surface-bound IgG and C3b molecules. Therefore, this facilitates bacterial penetration into the surrounding tissues and results in diminished uptake of the bacteria by neutrophils (96). Beside *S. aureus*, Streptococci also secrete a number of molecules that cleave IgG and C3 (97–100).

### 2.3 Evading neutrophil killing

The neutrophil granules contain an arsenal of weapons that can kill bacteria. Reactive oxygen species (ROS) are a key component of the neutrophil granule that kill bacterial pathogens. It is not surprising that bacteria have developed an array of mechanisms that evade ROS mediated killing, e.g. by inhibition of ROS production or misdirection of the oxidase complex away from phagosomes. *S. aureus* shields itself against ROS via their characteristic golden pigment, staphyloxanthin, that possess antioxidant properties and provides resistance to killing by peroxide and singlet oxygen (101). This function is confirmed by both *in vivo* and *in vitro* studies. In addition to staphyloxanthin, several staphylococcal scavengers like catalase (KatA) and superoxide dismutases (sodA and sodM) also contribute in the resistance against ROS (102, 103). Recently, Staphylococcus peroxidase inhibitor (SPIN) was identified by the high throughput selection strategy phage display as an MPO binder. Through its specific intercalation into the active site of MPO, SPIN proved an effective inhibitor of MPO mediated hypochlorous acid generation and killing (104).

Besides ROS, antimicrobial peptides (AMPs) and enzymes in the phagolysosome act to facilitate the killing and degradation of ingested microbes. There are two major AMP families in mammals: the cathelicidins (of which LL- 37 is the sole member in humans) and the defensins (the α and β families). Prototypical AMPs have a net positive charge to facilitate interaction with the net negative charge of bacterial surfaces. *S. aureus* resistance against AMPs is mediated by positive-charge modifications of the cell wall. D-alanine modifications of wall teichoic acid (WTA) and leipoteichoic acid (LTA) are well-recognised as providing these otherwise anionic structures with a more neutral charge thereby subverting AMP killing (105). *S. aureus* is also capable of modifying the cell membrane. The *S. aureus* membrane, like all bacteria, mainly consists of the negatively charged phosphatidylglycerol (PG), however, the enzyme MprF modifies PG via the addition of L-lysine. This modification renders PG less negative in charge and results in repelling of cationic AMPs. Therefore, an MprF mutant strain is killed considerably faster by human neutrophils and exhibits attenuated virulence in mice (106, 107). Other resistance mechanisms against AMPs are also employed by *S. aureus*. For example, they can secrete aureolysin, a metalloprotease, which is able to cleave the human cathelicidin LL-37 (108). In addition to AMP resistance, *S. aureus* also developed mechanisms to evade targeting by other neutrophil granule constituents. For instance, NSPs from secondary granules are targeted by Eap, and its two structural homologues EapH1 and EapH2, secreted molecules that inhibit NSP mediated degradation of *S. aureus* immune evasion molecules (109).

As discussed in part 1, neutrophil NETosis is another important strategy of neutrophils to combat invading pathogens. However, bacterial secreted endonucleases destroy the DNA network and enable them to be liberated from the traps. An alternative NET escape mechanism of *S. aureus* is by converting NETs to deoxyadenosine, which induces macrophage cytotoxicity. Two secreted proteins, nuclease and adenosine synthase are required for this induced host cell death (110). Besides that, bacteria are also able to produce DNA binding proteins that can block NETs formation, such as the multifunctional protein Eap (111).

### 2.4 Bacteria directly attack neutrophils

Although the previous discussed bacterial mechanisms are all focused around defending against host attacks, bacteria are also capable of attacking. The attack is accomplished by employing toxins that lyse the neutrophils both before and after phagocytosis. *S. aureus* produces toxins that include amphipathic-phenol-soluble modulins (PSMs) and bicomponent β-barrel pore-forming leukocidins. PSMs, at micromolar concentrations, represent the most abundant cytolytic peptides (112), although at nanomolar concentrations they also are potent neutrophil







chemoattractants sensed by the neutrophil receptor FPR2 (113). The lipoproteins present in serum/plasma can fully block PSMs functions in both the cell lysis and FPR2-mediated neutrophil activation (114). Of the leukocidens, five have been described for *S. aureus*: Panton-Valentine leukocidin (PVL), hemolysin-gamma (Hlg), leukocidin E/D (LukED), leukocidin G/H (LukGH), and leukocidin M/F-like (LukMF). The species specificity of these bicomponent toxins is most likely determined by the interaction with host GPCRs, as demonstrated for CCR5 and LukED (115) and for C5aR and CD45 for PVL and HlgCB (116–118). By targeting GPCRs, *S. aureus* has a high chance to attack phagocytes before they have been phagocytosed. A more detailed description of all the toxins can be found in numerous reviews (119–122).

A more subtle way of attacking the host immune system is by manipulating the host response. Invading bacteria are capable of targeting inhibitory receptors on immune cells that bear immunoreceptor tyrosine-based inhibitory motifs (ITIM) (123, 124). These receptors, upon binding of their ligand, down regulated activating signals these cells receive (125, 126). By binding these receptors bacteria have been found to suppress TLR2-mediated responses, phagocytosis, and oxidative burst (127, 128). For example, *S. aureus* can target the murine specific ITIM motif containing receptor PIR-B through LTA and possibly another *S. aureus* specific ligand. This results in dampening of the immune responses against *S. aureus* (129). Additionally, *S. aureus* binds the human homolog, although the functional consequences of this interaction remains poorly characterized.

In conclusion, bacteria have developed many different strategies to combat with the humane immune system. These strategies have been very well described for the pathogen *S. aureus*, and typically involve defense against host killing, but also specific attack strategies that suppress or reduce immune cell activity. In order to combat these versatile pathogens it is important to fully understand the immune evasion strategies. This, however, requires many additional studies to identify novel interactions, to characterize the molecular basis of these interactions and to define the functional consequences.

## Part 3 Future perspectives

In order to develop novel strategies to prevent or treat infection or inflammation, we need better knowledge of innate immune responses and host-pathogen interactions. This includes improved characterization of the role of immune receptors in neutrophil biology, defining the full repertoire of bacterial immune evasion mechanisms and understanding whether these mechanisms and interactions can be targeted or exploited in future therapeutics.

### 3.1 Improved understanding of neutrophil biology

Neutrophils play an indispensable role in the host defense against bacteria, but have also been shown to be important in other inflammatory diseases such as autoimmunity and cancer. As described in part 1, neutrophils are equipped with a large amount of immune modulating receptors that have the abilities to fulfill their effector functions by activation, suppression or both. Why do neutrophils utilize so many receptors? It is most likely that neutrophils must recognize and response to many different internal and external environmental conditions that inform the neutrophil to remain resting, to become ready for combat or to destroy invading microbes. To really understand the various functions of the individual immune modulating receptors in both homeostatic and the relevant pathologic conditions, one needs to know where and when the receptor is expressed and its functions. The identification and characterization of new receptors can provide a better understanding of the neutrophil role in health and disease situations.

### 3.2 Identification and characterization of novel immune evasion molecules

Many bacterial species, both primary pathogens and commensal bacteria, have evolved an arsenal of immune evasion molecules. The fundamental mechanism of these molecules is similar, i.e. to avoid detection and/or killing by the host. These molecules are interesting targets for alternative therapeutics and vaccines. Especially in an era of increasing bacterial antibiotic resistance, in particular among commensal bacteria that are opportunistic pathogens. Therefore, the identification and characterization of immune evasion molecules is extremely important. Fortunately, high throughput screening methods based on massively parallel sequencing, such as phage display and transposon sequencing (Tn-seq) (130, 131), allow researchers to efficiently identify new immune interaction molecules from different bacterial species.

In this thesis, we use the secretome phage display to identify new molecules that interact with the immune system. For phage display, bacterial genomic DNA is randomly fragmented and ligated into a phage display vector. Phages will therefore express small parts of secretome proteins on their surface. This phage library is subsequently used to select for interaction with a target, e.g. host immune components.

### 3.3 Towards novel therapeutic strategies

The discovery of antibiotic drugs during the last century dramatically decreased the mortality and morbidity of infectious diseases. Today, increasing resistance to these antimicrobial drugs threatens the effectivity of antibiotics. This calls for new approaches to extend the possibilities for therapeutic prevention and intervention







of infections. Vaccine efficacy is severely hampered by existing immune evasion molecules. Antibodies, raised by the vaccine, simply cannot function properly when immune evasion molecules resist the influx of effector systems as neutrophils and down modulate the complement system. Inclusion of immune evasion molecules in vaccines is a novel approach to increase the efficiency of vaccines. Using identified immune evasion proteins in the vaccine will raise blocking antibodies that inhibit the inhibitor. Including immune evasion molecules of bacteria in vaccines may enhance anti-microbial defenses and prevent the invasion of pathogenic bacteria.

Next to host defense, it has become clear that the immune system also plays a major role in pathogenesis of inflammatory diseases like sepsis, auto immune diseases, degenerative diseases, cardiovascular diseases and cancer. These diseases are major challenges of modern medicine. Research of the mechanisms underlying these diseases has identified numerous new targets for potential therapeutic intervention. The next step is to find new compounds acting on these targets in order to beneficially modulate immune responses. This research project provides an unique way to identify molecules with immune modulating properties from bacterial origin. Understanding the mechanism behind these molecules will provide a deeper understanding of the immune system and mechanisms of immune modulation and will teach us more about the pathogenesis of infectious diseases. Eventually this will lead to the development of new therapeutic strategies in inflammatory and infectious disorders.

## Aim and scope of this thesis

The aim of this thesis is best described by Sun Tzu, a 6$^{th}$ century B.C. Chinese general. In his book 'The Art of War' he wrote "If you know both yourself and your enemy, you can win numerous battles without jeopardy". This also holds true for battling bacterial infections. This thesis describes our efforts in exploring the interactions between the host neutrophil and the enemy bacteria. In chapter 2, we characterized LILRs as potent modulators of FcγR-mediated effector functions of neutrophils, including ROS production, phagocytosis and killing of microbes. In chapter 3, we set out a high-throughput screening method, the secretome phage display, to discover novel *S. aureus* immune interaction proteins targeting human neutrophils. We identified and characterized the staphylococcus superantigen like protein 13 (SSL13), which acts as a neutrophil activator via human FPR2. In chapter 4, using the same screening method as chapter 3, we identified and characterized a new immunoglobulin binding protein from the gut microbiome. In chapter 5, we exploit CHIPS as a potential candidate to treat C5aR mediated diseases in humans. Finally, we discuss the role of these new identified immune interaction proteins and the role of diverse neutrophil receptors, as well as their therapeutic implications in Chapter 6.

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CHAPTER 2

# Leukocyte immunoglobulin-like receptors (LILRs) modulate Fcγ receptor mediated functions of neutrophils

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## Abstract

Neutrophils are critical to the generation of effective immune responses and for killing invading microbes. Immune receptors provide an important mechanism to generate, shape and modulate neutrophil activity and their antimicrobial effector functions. The leukocyte immunoglobulin-like receptors (LILRs) are immunomodulatory receptors expressed on a variety of immune cells. Whether LILRs are involved in modulation of microbial clearance is unclear. Here, we demonstrate that primary human neutrophils express LILRA2, LILRA6, LILRB3 and LILRB4 at detectable levels. Stimulation of inhibitory LILR (LILRB3 or B4) that bear ITIM suppressed Fcγ receptor-mediated activation of neutrophils. We demonstrate that certain inhibitory LILRs can modulate key effector functions against antibody-opsonized microbes, including production of reactive oxygen species (ROS), phagocytic uptake and killing. Notably, inhibitory LILRs had different abilities to modulate these effector functions - LILRB3 or LILRB4 modulated phagocytic uptake of antibody-opsonized microbes, whilst only LILRB3 modulated their killing. This suggests that LILR family of receptors provide an important checkpoint to control antimicrobial effector functions of neutrophils, and could be a target for future immunomodulatory therapeutics.



**Keywords**

Leukocyte immunoglublin-like receptor, modulation, neutrophil, inhibitory, ILT, CD85








## Introduction

Neutrophils provide the first-line of defense against invading microbes, and have key roles in orchestrating inflammation (1). When mobilized to sites of infection, neutrophils recognize pathogens, or opsonized pathogens, through surface receptors. Pathogens are killed through a combination of effector functions including phagocytosis, and subsequent exposure to an arsenal of antimicrobial compounds, or through degranulation, the release of reactive oxygen species (ROS) and other antimicrobial compounds (2). However, when mobilized at the wrong time and place, the potent effector functions of neutrophils can damage the host and cause inappropriate inflammatory responses. Accordingly, neutrophils must be tightly regulated in order to protect the host and to avoid excessive immune responses (3).

Immune receptors have critical roles in regulating immune cell activation and effector functions. A diverse range of surface receptors for recognizing microbial infection and for maintaining balanced responses has been identified on neutrophils (3, 4). Binding of ligands to activatory receptors, including the Fc receptors (FcRs), G protein-couple receptors (GPCRs) and Toll-like receptors (TLRs), induces activation and effector functions. The activation threshold may be increased by the expression of inhibitory receptors, that upon stimulation suppress cell activity through opposing signaling mechanisms and thereby ensure that balanced neutrophil responses are generated (5). FcRs recognize antibody-opsonized pathogens, and cross-linking leads to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails (FcγRIIA or CD32a) or on Fc common γ chain cytoplasmic tail (FcγRIA or CD64, and FcγR or CD89)(4). ITAM phosphorylation leads to recruitment of Syk tyrosine kinase, activation of Syk, several tyrosine kinase substrates and subsequent downstream signaling (6). FcRs mediate a plethora of functions including phagocytosis, degranulation and cytokine release. Activation of neutrophils through ITAM signals can be suppressed by the activation of receptors that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails. Inhibitory receptors opposing these receptors, include leukocyte-associated Ig-like receptor 1 (LAIR-1) and signal inhibitory receptor on leukocytes 1 (SIRL-1). Upon ligand binding ITIMs become phosphorylated, leading to docking of SH2 domain containing tyrosine phosphatases such as SHIP-1, Shp1 and Shp2, and suppression of activatory downstream signaling (7). Consequently, activatory and inhibitory receptors are important mechanisms that modify activation thresholds and ensure the generation of appropriate immune responses.

The human leukocyte immunoglobulin-like receptors (LILRs), also known as immunoglobulin-like transcripts (ILTs), are a family of 11 receptors that have a

powerful ability to modulate immune cell activity, function and phenotype (8, 9). All LILRs possess an extracellular domain containing Ig-like domains and a transmembrane domain, except for LILRA3 that is soluble. LILRA1, A2, A4, A5 and A6 possess short cytoplasmic tails, and signal through the ITAMs in the Fc common γ chain cytoplasmic tail. LILRB1 to B4 possess long cytoplasmic tails containing ITIMs. Consequently, binding of ligands to LILRAs or LILRBs can lower or enhance the immune activation threshold. Though certain LILRs are known to be expressed on neutrophils (3, 10, 11), and there is emerging evidence that they can modulate neutrophil activation, robust knowledge of expression and functions of all LILRs on neutrophils is lacking (12). The best-characterized examples are, LILRA2, which has been demonstrated to be induce neutrophil activation upon recognition of microbially cleaved immunoglobulins (13) and LILRB2, which has been characterized as a modulator of neutrophil phagocytosis and degranulation (11). Additionally, the mouse orthologue paired-immunoglobulin-like receptor (PIR)-B regulates respiratory burst in neutrophils (14). This suggests LILRs have important roles in generating and maintaining balanced neutrophil responses.

In this study, we characterize LILRs as potent modulators of FcγR-mediated effector functions of neutrophils including ROS production, phagocytosis and killing of microbes. Our data demonstrates that LILRs might be considered as the targets of therapeutics to modify neutrophil functions and immune responses during disease situations.

## Materials and methods

### Neutrophil isolation

This study was carried out in accordance with the recommendations o fMETC-protocol 07-125/C approved on 1 March 2010 from the medical ethics committee of the University Medical Center Utrecht with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the 'medical ethics committee of the University Medical Center Utrecht'. Neutrophils were isolated by Ficoll/Histopaque centrifugation as previously described (15).

### Antibodies

Neutrophils were incubated with a selection of the following antibodies;- anti-LILRA1 (clone 586326, R&D Biosystems), anti-LILRA2 (clone 600007, R&D Biosystems), anti-LILRA6 (clone 921330, R&D Biosystems), anti-LILRB1 (clone GHI/75, ITK Diagnostics), anti-LILRB2 (clone 42D1, Santa Cruz), anti-LILRB3 (clone 222821, R&D Biosystems), anti-LILRB4 (clone ZM3.8, BD Biosciences) and isotype control human IgG1 (Sanquin). Anti-mouse-IgG1-PE (Agilent) or anti-rat-IgG-PE (Jackson ImmunoResearch) was used for flow cytometry. Anti-CD35-PE (clone E11, BD Biosciences) and anti-CD63-PE (clone 435, ImmunoTech) were used as detection of secretory vesicle and granule exocytosis. Rabbit anti-HSA IgG fraction (Sigma) was used for ROS production using HSA immune complexes. *S. aureus* was opsonized with anti-WTA or anti-HIV gp120 that we expressed and purified in a eukaryotic expression system. Briefly, gBlocks containing human IgG1 heavy (HC) and light chain (LC) constant regions were with cloned into pcDNA3.4 vector. Next, gBlocks containing variable heavy (VH) and light chain (VL) sequences were cloned into pcDNA3.4-HCIg-hG1 and pcDNA3.4-LCIg-hk), as well as upstream KOZAK sequence and HAVT20 signal peptide, using NheI and BsiWI as the 3'cloning sites for VH and VL, respectively, in order to preserve the immunoglobulin heavy and kappa light chain amino acid sequence. VH and VL sequences were derived from previously described antibodies directed against staphylococcal wall teichoic acid GlcNac-beta (anti-WTA clone 4497; based on WO2014/193722)) and against HIV gp120 (clone b12) which served as control (16, 17, 18). Antibodies were expressed as IgG1/Kappa in Expi293F cells (Life Technologies) essentially as described before (19) and purified by affinity chromatography (ÄKTA Pure, GE Healthcare Life Sciences) using a Protein A column (GE Healthcare Life Sciences). Elutions into 0.1 M Citric acid pH 3.0 were dialysed against PBS at 4°C. Hamster anti-mouse-CD3e clone 145-2C11 (BD Biosciences) was used to test CD3 mediated GFP expression in 2B4T cells.

### Construction of lentiviral vector containing LILRCD3ζ fusion protein inserts

DNA fragments containing the coding domain sequence (CDS) of the extracellular and transmembrane domains of LILRs and the CD3ζ cytoplasmic tail were synthesized by Integrated DNA Technologies, and were ligated into a dual promoter lentiviral vector (BIC-PGK-Zeo-T2a-mAmetrine; RP172 derived from no.2025. pCCLsin.PPT.pA.CTE.4 .-scrT.eGFP.mCMV.hPGK.NG-FR.pre, as previously described (20)) via Gibson reaction. Vectors containing correct inserts were confirmed by colony PCR and sequencing. RP172 vector contains human EF1A promoter for potent expression of the cloned downstream gene and the PGK promoter which controls expression of mAmetrine and selection marker for zeocin resistance.

### Generation of lentiviral particles and transduction into cells

HEK293T cells were seeded at 6.25 x $10^4$ cells/ml in RMPI1640 + 10% FCS+ 100µg/ml penicillin and 100µg/ml streptomycin and cultured at 37°C + 5% $CO_2$ for 24 hours. 50µl RMPI1640 and TransIT-LT1 transfection reagent (MirusBio, USA) were mixed and incubated for 5 minutes at room temperature. Subsequently, 0.25µg packaging mix

(containing equal parts of pVSV-G, pMDL and pRSV vectors) and 0.25 µg of RP172 vector were added, mixed and incubated at room temperature for 20 minutes. This mixture was added to 1 ml of HEK293T cells and incubated at 37°C and 5% $CO_2$ for 72 hours. 100 µl of the supernate containing lentivirus particles was added to 500 µl of 1 x $10^5$ cells/ml of 2B4T cells in RPMI 1640 supplemented with 10% FCS+ 100µg/ml penicillin, 100 µg/ml streptomycin and 8 µg/ml polybrene. After centrifugation at 2000 rpm for 90 minutes at 33°C, 500 µl of RPMI 1640 supplemented with 10% FCS+ 100µg/ml penicillin, 100 µg/ml streptomycin was added and cells were incubated at 37°C + 5% $CO_2$. After 72 hours, an additional 5 ml of RPMI 1640 supplemented with 10% FCS+ 100µg/ml penicillin, 100 µg/ml streptomycin and 400 µg/ml zeocin was added to cell cultures to select for transfectants. Single cell cultures were formed and LILR expression measured using anti-LILR mAb and flow cytometry analysis.

### Induction of GFP expression in 2B4T cells

The wells of 96-well plate were coated overnight at 4°C with 40 µl of 15 µg/ml anti-CD3, anti-LILR or isotype control mAb. After washing, 200 µl of 2B4T cells were seeded at 2.5 x $10^5$ cells/ml in RPMI + 10% FCS + 100 µg/ml penicillin and 100 µg/ml streptomycin and cultured at 37°C + 5% $CO_2$ for 18 hours. After washing and resuspension of cells in PBS, fluorescence of 2B4T cells was measured by flow cytometry.

### Measurement of LILR expression

To analyze the expression of LILR at the cell-surface, isolated primary neutrophils (35 µl of 5 x $10^6$ cells/ml) were incubated with 5 µg/ml of anti-LILRA1, anti-LILRA6, anti-LILRB1, anti-LILRB2, anti-LILRB3 or anti-LILRB4, or isotype matched, antibodies for 1 hr at 4°C. After washing, neutrophils were incubated with 5 µg/ml of PE-conjugated goat anti-mouse IgG (for anti-LILRA1, anti-LILRA2, anti-LILRA6, anti-LILRB1, anti-LILRB3 and anti-LILRB4) or goat anti-rat IgG (for anti-LILRB2) antibodies for 1 hr at 4°C. After washing, neutrophils were fixed in 150 µl of 1% PFA. Expression data was collected for 10,000 events by flow cytometry, and analysis performed in FlowJo. To stimulate exocytosis of secretory vesicles, neutrophils were incubated at 37°C for 60 minutes. To stimulate additional exocytosis of granules, neutrophils were at 37°C for 60 minutes in the presence of 1µM fMLP and 10 µg/ml cytochalasin B.

### ROS production induced through HSA immune complexes

Wells of a 96-well white plate were coated overnight at 4°C with 20 µg/ml of HSA in carbonate buffer pH 9.5, washed with PBS, blocked with 10% FCS for 1 hr at 37°C, washed with PBS, and incubated with 4 or 0.4 µg/ml rabbit anti-HSA (Sigma Aldrich) for 1 hr at 37°C and washed with PBS. Primary neutrophils (50 µl of 1.25 x $10^6$) were







pre-treated in the presence or absence of 5 µg/ml anti-LILR or isotype matched control for 1 hr at 4°C. After washing, neutrophils were re-suspended in 100 µl of IMDM media + 0.1% HSA and then added to wells of the coated 96-well plate containing 100 µl of luminol balanced salt solution (LBSS) + 0.1% HSA pre-warmed at 37°C. Luminescence, and the production of ROS, was measured immediately at 2 minute intervals on a CLARIOstar. The level of ROS production was determined as area under the curve, with background subtraction of neutrophils only, during 60 minutes.

### ROS production induced through *S. aureus*

*S. aureus* Wood strain was cultured in Todd-Hewitt broth media at 37°C until $OD_{600}$ = 1.00, and diluted in PBS + 0.1% HSA to 2 x $10^8$ CFU/ml. Bacteria were incubated with 10 µg/ml anti-WTA IgG1 for 15 minutes at 37°C. 25 µl of opsonized *S. aureus* were added to wells of a plain 96-well white plate. Primary neutrophils (5 x $10^6$ cells/ml) were pre-treated in the presence or absence of 5 µg/ml anti-LILR or isotype matched control for 1 hr at 4°C. After washing, neutrophils were re-suspended in 100 µl of LBSS media + 0.1% HSA and then added to wells of a white 96-well plate containing 25 µl of opsonized *S. aureus* Wood pre-warmed at 37°C. Luminescence, and the production of ROS, was measured immediately at 2-minute intervals on a CLARIOstar. The level of ROS production was determined as area under the curve, with background subtraction of neutrophils only, during 60 minutes.

### Binding and phagocytosis of *S. aureus*

*S. aureus* Wood strain was labelled with FITC by culturing the strain in 5 ml of Todd-Hewitt browth media at 37°C until $OD_{600}$ = 1.00, and after centrifugation for 10 min at 4000 rpm, the bacterial pellet was re-suspended in 5 ml PBS containing 0.5 mg/ml FITC and incubated for 30 minutes on ice and in the dark. Bacteria were washed 3 times in PBS. To opsonize FITC-labelled *S. aureus*, 1 x$10^8$ CFU/ml were incubated with 5% heat-inactivated serum for 30 minutes at 4°C. Primary neutrophils (5 x $10^6$ cells/ml) were pre-treated in the presence or absence of 5 µg/ml anti-LILR or isotype matched control for 1 hr at 4°C. Neutrophils were re-suspended in 100 µl of RPMI 1640 media + 0.1% HSA after washing, and incubated with 5 x $10^7$ CFU/ml of opsonized FITC-labelled *S. aureus* for 1 hr at 4°C (*S. aureus* binding) or 37°C (*S. aureus* phagocytosis). To stop phagocytosis, neutrophils were washed in 100 µl of ice-cold IMDM + 0.1% HSA and fixed in 150 µl of 1% PFA. Expression data was collected for 10,000 events by flow cytometry, and analysis performed in FlowJo.

### Killing of *S. aureus*

*S. aureus* Wood strain was cultured in LB media at 37°C until $OD_{600}$ = 0.40, washed 3 times and resuspended in RPMI + 0.05% HSA at 1 x $10^8$ CFU/ml. In a siliconized microtube, 85 µl of sterile neutrophils (1 x $10^7$ cells/ml) was mixed with 10 µl of *S. aureus* Wood strain and 5 µl of heat-inactivated human serum, and incubated at 37°C for 2 hrs. Reactions were stopped by the addition of 900 µl of ice-cold 0.3% saponine, and serial dilutions plated onto Todd-Hewitt agar plates to enumerate CFU/ml after incubation at 37°C overnight.

## Results

### LILRs are expressed on neutrophils

The expression of LILRs on neutrophils remains poorly characterized (12). We investigated LILR expression on primary neutrophils using LILR-specific antibodies and flow cytometry. Neutrophils were isolated on Ficoll/Histopaque gradients with > 98% purity and 99% viability (21). Data from 8 healthy human donors was analyzed as relative MFI in comparison to neutrophils stained with isotype control mAb. The analysis indicated that LILRA1 (relative MFI = 1.47, $p < 0.05$), LILRA2 (relative MFI = 2.35, $p < 0.01$), LILRA6 (relative MFI = 3.72, $p < 0.05$), LILRB1 (relative MFI = 1.40, $p < 0.05$) and LILRB3 (relative MFI = 7.57, $p < 0.01$) were expressed at the surface of resting neutrophils. Given that different antibodies bind their respective antigens with different affinities and avidities, it is not possible to directly compare expression of different LILRs. Expression of LILRB1 and LILRB2 were not convincingly detected at the surface of resting neutrophils across multiple donors. We detected elevated MFI for LILRB4 compared to isotype controls for 4/8 donors. Figure 1B shows the data of 1 donor. Our data is consistent with previous detected expression of LILR on the surface of resting neutrophils (22), but notably we were unable to detect LILRB1 or LILRB2 expression with the mAb implemented in our assays.

Immune receptors can also be expressed in neutrophil secretory vesicles or granules, whereby neutrophil activation results in release and increased expression of the immune receptor at the cell surface. Alternatively, surface expression of receptors may decrease following neutrophil activation. To confirm whether LILRs are localized in secretory vesicles, we compared receptor expression between resting neutrophils and those incubated at 37°C, which is sufficient to mobilize the release of secretory vesicles and up-regulation of CD35 expression (23). Indeed, we detected a 2-fold increase in surface expression of the secretory vesicle marker CD35 ($p$ <0.01), but not of the granule marker CD63 (Figure 2A). There was no concomitant increase in LILR expression suggesting that LILRs are not localized within secretory vesicles (Figure 2B). Neutrophils can be stimulated to release the







granules by incubation at 37°C in the presence of fMLP and cytochalasin B (23). We detected a 4-fold increase in CD63 surface expression upon incubation of neutrophils at 37°C in the presence of 1 µM fMLP and 10 µg/ml cytochalasin B (Figure 2A), and also detected a 2-fold decrease in CD35 surface expression. To determine whether LILRs are localized in granules, we compared LILR surface levels between neutrophils incubated at 37°C in the presence of 1 µM fMLP and 10 µg/ml cytochalasin B (granules and secretory vesicles released) against neutrophils incubated at 37°C (secretory vesicles released). Whilst there was evidence to suggest LILRB2 ($p < 0.05$) expression increased during exocytosis of granules (Figure 2B), in agreement with previous findings (11), there was no evidence that surface expression of other LILR increased. Collectively, this data demonstrates that LILRs are localized at the surface, and in the granules, of resting neutrophils.

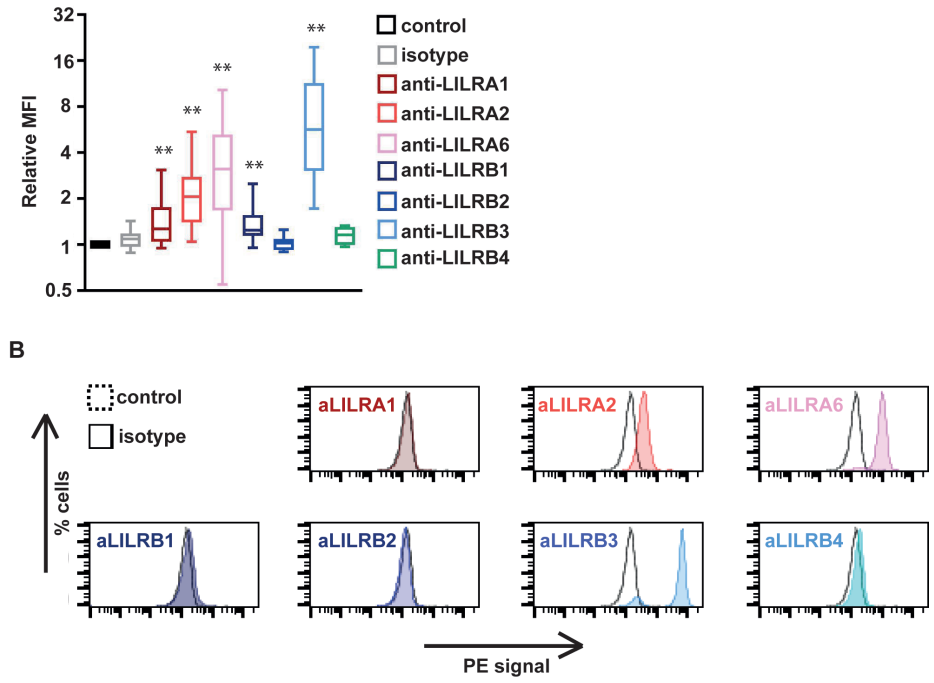


**Figure 1. LILRs are expressed by neutrophils.** A) Neutrophils from 8 donors were stained with anti-LILR or isotype control mAb, followed by anti-IgG-PE. The relative mean fluorescence intensity (MFI) was calculated by comparing the MFI of neutrophil groups against the positive control (POS control). Relative MFI was compared between neutrophils stained with anti-LILR mAb versus isotype mAb using Wilcoxon signed rank test, * = $p < 0.05$, ** = $p < 0.01$. B) Representative data of LILR expression on neutrophils from 1 donor.

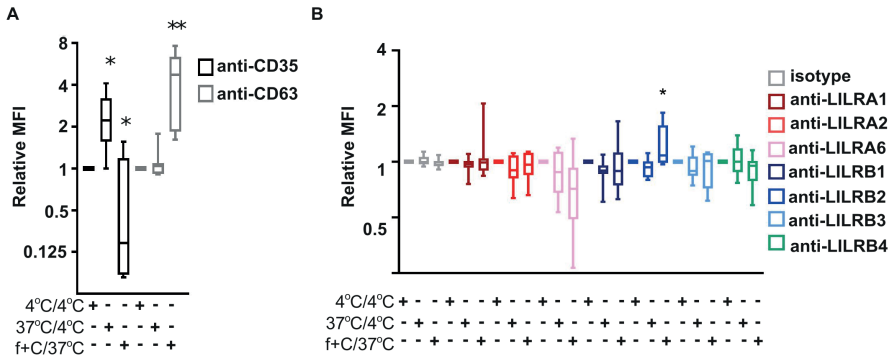


**Figure 2. LILR expression at the neutrophil surface upon exocytosis of secretory vesicles and granules.** Neutrophils from 8 donors were incubated at three conditions (i) 4°C, (ii) 37°C or (iii) 37oC in the presence of 1 μM fMLP and 10 μg/ml cytochalasin B for 60 mins. A) Neutrophils were subsequently stained for secretory vesicle (CD35) and granule specific (CD63) markers, and fluorescence was measured by flow cytometry. MFI of neutrophil populations was normalized against unstained control neutrophils within each condition. Background level of CD35 or CD63 expression on resting neutrophils was normalized to 1 (4°C/4°C). Relative surface expression of CD35 or CD63 was calculated at 37°C in comparison to 4°C for secretory vesicles release, and at 37°C in presence of fMLP and cytochalasin B (f+C) in comparison to 37°C for granule release. Relative MFI differences between compared between anti-CD35 or anti-CD63 stained populations by Wilcoxon signed-rank test, * = $p < 0.01$, ** = $p < 0.05$. B) Neutrophils were subsequently stained for LILR, and fluorescence was measured by flow cytometry. MFI of neutrophil populations was normalized against unstained control neutrophils within each condition. Background level of anti-LILR mAb or isotype mAb on resting neutrophils was normalized to 1 (4°C/4°C). Relative surface expression was calculated at 37°C in comparison to 4°C for secretory vesicles release, and at 37°C in presence of fMLP and cytochalasin B (f+C) in comparison to 37°C for granule release. Relative MFI differences were compared between anti-LILR and isotype mAb groups by Student *t*-test.

## LILR stimulation inhibits FcγR-mediated production of ROS

Neutrophils mainly express three types of FcγR, the medium affinity receptors FcγRIIa (CD32a) and FcγRIIIb (CD16b), and the high-affinity FcγRI (CD64) following activation (4). Stimulation of these receptors induces ROS production in neutrophils, and this can be inhibited by stimulation of ITIM-bearing inhibitory receptors such as SIRL-1 or CD300a (24, 25). To determine whether LILRs could modulate FcγR mediated ROS production, primary neutrophils were pretreated with LILR-specific antibodies and then stimulated using plate-bound HSA immune complexes formed with anti-HSA IgG. The anti-LILRB1 GHI/75, anti-LILRB2 42D1, anti-LILRB4 ZM3.8 have been described as efficient LILR agonists (12, 26, 27). Information on the activating abilities of other mAb was absent. The agonistic or antagonistic effects of receptor ligands has been elucidated for LILRs, and other receptors, using Nuclear Factor of Activated T cell–Green Fluorescent Protein (NFAT-GFP) reporter systems in mouse 2B4T hybridoma cells (28). To determine the agonistic properties of other anti-







LILR mAb, we constructed 2B4T cell lines that expressed LILR-CD3ζ fusion proteins and tested the ability of anti-LILR mAb to induce NFAT-GFP expression, using anti-LILRB1 on a LILRB1CD3ζ cells as a positive control. Surface expression of fusion proteins, composed of the extracellular and transmembrane domains of LILRA1, A2, A6, B1 and B3 and the CD3ζ cytoplasmic tail, were detected in transduced cells but not controls (Supplementary Figure 1A). GFP-expression was induced in each 2B4T cell population through native mouse CD3 using anti-CD3 mAb but not in buffer controls, indicative that CD3ζ cytoplasmic tail can be activated and induce NFAT signaling pathway (Supplementary Figure 1B). As expected, anti-LILRB1 GHI/75, but not isotype, mAb induced a significant ($p < 0.05$) increase in the percentage of GFP positive LILB1CD3ζ expressing 2B4T cells, but not control cells (Figure 3A and B). Additionally, anti-LILRA1 586326 mAb ($p < 0.05$), anti-LILRA6 287219 mAb ($p < 0.01$) and anti-LILRB3 222821 mAb ($p < 0.01$) induced significant GFP expression in 2B4T cells expressing LILRA1CD3ζ, LILRA6CD3ζ and LILRB3CD3ζ fusions, respectively, but not control cells (Figure 3A and B). Notably, anti-LILRA1 induced lower levels of GFP expression than anti-LILRA6, anti-LILRB1 and anti-LILRB3 mAbs. Further analysis would be required to compare the strength of agonistic properties. Nonetheless, the data demonstrates that these mAb can act as LILR agonists. As the extracellular Ig-like domains of LILRA6 and LILRB3 are highly homologous (29), we tested whether the anti-LILRA6 and anti-LILRB3 were cross-reactive. We found that anti-LILRA6 and anti-LILRB3 both detected LILRA6 and LILRB3 fusion proteins on the surface of 2B4T

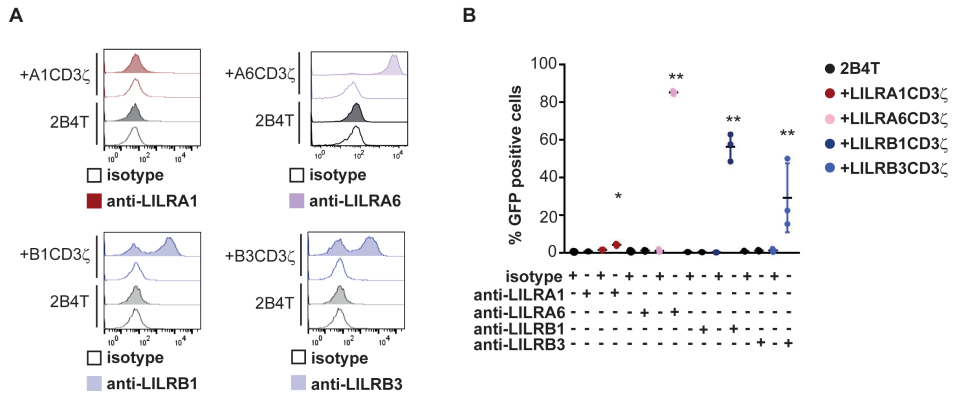


**Figure 3. anti-LILR mAb are LILR agonists.** (A) GFP-expression in 2B4T cells expressing LILRCD3ζ (A1, A6, B1 or B3) fusion proteins or control 2B4T cells upon incubation in wells coated with anti-LILR or isotype mAb for 18 hours. Representative plots are shown. (B) anti-LILR mAb (LILRA1, LILRA6, LILRB1 and LILRB3) induced a significant increase in GFP positive 2B4T cells expressing their respective LILRCD3ζ (A1, A6, B1 or B3) fusion proteins in comparison to control 2B4T cells. A paired Student *t*-test was used to compare % of GFP positive cells between anti-LILR or isotype control mAb (*n* =3).

cells (Supplementary Figure 2A). Given the cross-reactivity, we hypothesized both mAb are able to serve as LILRA6 and LILRB3 agonists. Indeed, anti-LILRA6 induced GFP expression in 2B4T cells bearing LILRA6CD3ζ and LILRB3CD3ζ,but not control 2B4T cells (Supplementary Figure 2). Similarly, anti-LILRB3 induced GFP expression in both cells transduced with LILRCD3ζ, fusions but not control 2B4T cells. This indicates the agonistic effects of these mAb are cross-reactive.

Neutrophils can be stimulated to produce significant levels of ROS through exposure to HSA and anti-HSA IgG immune complexes, compared to unstimulated neutrophils (Figure 4A and 4B) (30). We next tested whether pre-treatment of neutrophils with LILR-specific mAb enhanced or suppressed ROS production, in comparison to neutrophils pre-treated with an isotype control mAb. As we could not detect expression of LILRB1 or LILRB2 on the surface of resting neutrophils with the mAb in this study, we did not include these mAb's in our assays. Pre-treatment of neutrophils with anti-LILR led to no significant reduction in the relative ROS production compared to isotype control mAb when an immune complex was formed with 4 µg/ml anti-HSA (Figure 4A and B). This suggests the activatory signal could not be modulated by LILR. However, pre-treatment of neutrophils with anti-LILRB3 and anti-LILRB4 led to a significant reduction in relative ROS production when the HSA immune complex was formed with 0.4 µg/ml, but not 4 µg/ml, anti-HSA IgG (Figure 4A and B). This suggests that LILRB3 and LILRB4 regulate FcγR mediated functions on neutrophils. In addition, pre-treatment of neutrophils with anto-LILRB3 or anti-LILRB4 but not their specific isotype control IgG suppressed FcγR mediated ROS production (Supplementary Figure 3A and B). Pre-treatment with anti-LILRA2 led to increased ROS production, though this was not significant. Collectively, the data suggests that LILR may fine-tune neutrophil activation.

Neutrophils bind antibody-opsonized bacteria through FcγRs and exert antimicrobial effector functions. We tested the hypothesis that LILR could modulate ROS production of neutrophils in response to opsonized *S. aureus*. ROS production was induced in neutrophils exposed to *S. aureus* opsonized with human anti-WTA IgG mAb, but not in neutrophils exposed to *S. aureus* opsonized with control anti-HIV IgG mAb (Figures 5A and 5B). Next, we compared ROS production between neutrophils pre-treated with anti-LILR or isotype control mAb upon exposure to anti-WTA IgG opsonized *S. aureus*. Pre-treatment of neutrophils with anti-LILRB3 inhibited ROS production induced by IgG-opsonized *S. aureus*. In contrast, other anti-LILR mAb nor isotype control mAb did ROS production (Figures 5B). This data further supports a role for LILR in modulation of FcγR-mediated neutrophil responses.







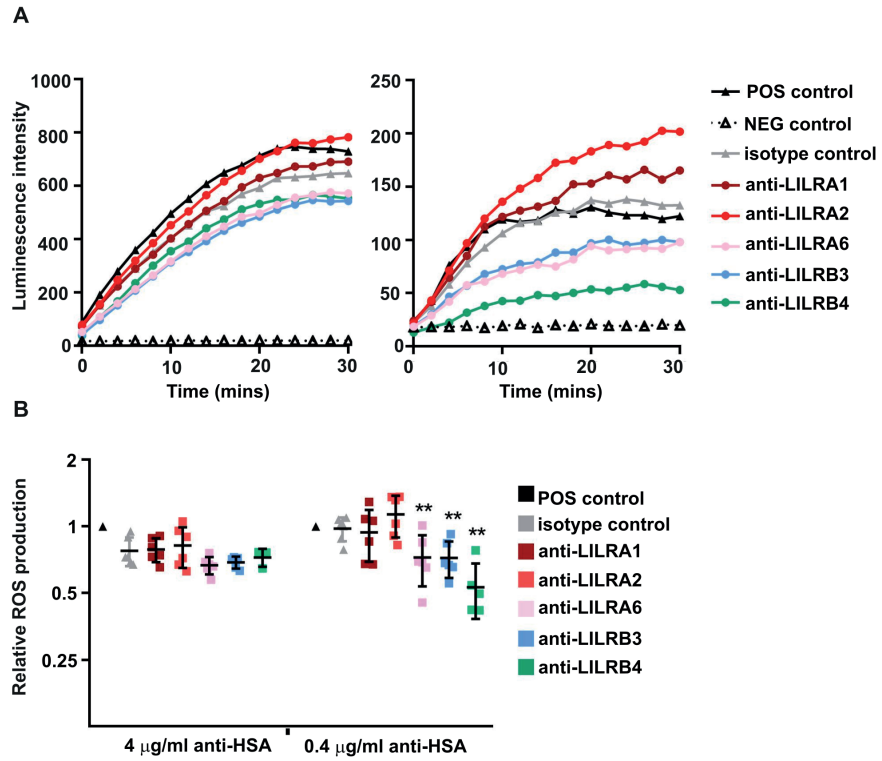


**Figure 4. LILRs modulate ROS production in neutrophils.** (A) Reactive oxygen species (ROS) production was stimulated with an HSA immune complex, formed with either 4 µg/ml or 0.4 µg/ml rabbit aHSA IgG (POS control), but not in unexposed neutrophils (NEG control). Neutrophils were pre-incubated with anti-LILR or isotype control mAb. Representative plots are shown. (B) Relative ROS production was calculated by comparing the area under the curve of neutrophil groups against the positive control (POS control). Relative ROS production was compared between neutrophils pre-treated with anti-LILR mAb versus isotype mAb using Student *t*-test ($n$ =6).

## LILR modulate FcR-mediated phagocytosis and killing of microbes

To further characterize LILR modulation of FcR-mediated effector functions, we measured the ability of neutrophils to bind and phagocytose fluorescently labeled antibody-opsonized *S. aureus* (31). In order to remove the effect of any complement mediated factors, we used 5% heat-inactivated serum to opsonize *S. aureus* in all experiments. After 1 hr incubation at 37°C in the presence of 5% heat-inactivated serum, 25.84% (standard deviation ± 4.05) of neutrophils were fluorescent, indicative that FITC-labeled *S. aureus* were bound at the cell surface or had been phagocytosed (Figure 5C). Next, we activated individual LILRs by pre-treatment with LILR-specific mAb, and measured whether this influenced phagocytic uptake

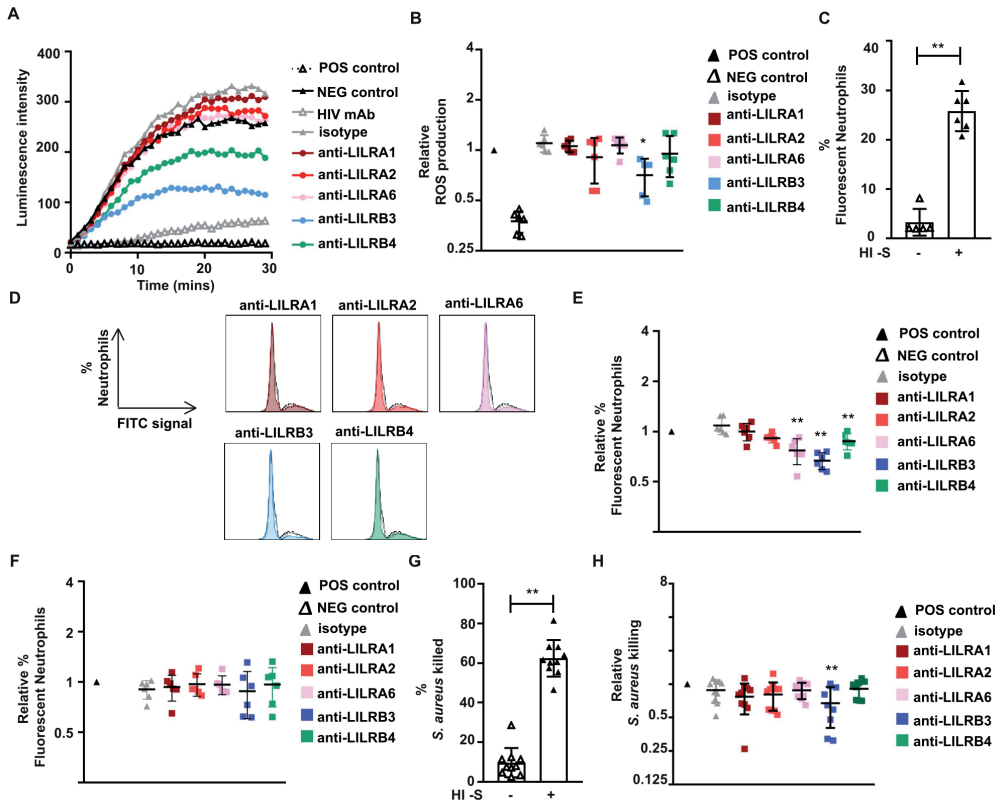


**Figure 5. LILRs modulate Fc receptor mediated antibacterial effector functions of neutrophils.** (A) FcγR-mediated reactive oxygen species (ROS) production by neutrophils was induced by exposure to aWTA IgG opsonized *S. aureus* (POS control), but not by exposure to aHIV IgG *opsonized S. aureus* (HIV mAb) or buffer control (NEG control). Pre-treatment of neutrophils with anti-LILRB mAb, but not anti-LILRA or isotype mAb, lead to decreased ROS production. A representative plot from 1 donor is shown. (B) Relative ROS production was calculated by comparing the area under the curve of neutrophil groups against the positive control (POS control). Relative ROS production was compared between neutrophils pre-treated with anti-LILR mAb versus isotype mAb using Mann Whitney test. (C) Phagocytosis of FITC-labeled *S. aureus* by neutrophils at 37°C in the presence or absence of 5% heat-inactivated serum, compared by Mann Whitney test (D) Phagocytosis of FITC-labeled *S. aureus* by neutrophils at 37°C. Pre-treatment of neutrophils with anti-LILRB3 mAb, but not isotype mAb, lead to decreased % of fluorescent neutrophils. (E) Phagocytosis of FITC-labelled *S. aureus* by neutrophils at 37°C in the presence of 5% heat-inactivated serum. Relative % of fluorescent neutrophils was calculated by comparing % of fluorescent neutrophils of neutrophil groups against the positive control (POS control). Relative % of fluorescent neutrophils was compared between neutrophils pre-treated with anti-LILR mAb versus isotype mAb using Mann Whitney test. Neutrophils with no FITC-labelled *S. aureus* = NEG control. (F) Binding of FITC-labeled *S. aureus* to neutrophils at 4°C in the presence of 5% heat-inactivated serum. Relative % of fluorescent neutrophils was calculated by comparing % of fluorescent neutrophils of neutrophil groups against the positive control. Relative % of fluorescent neutrophils was compared between neutrophils pre-treated with anti-LILR mAb versus isotype mAb using Mann Whitney test. Neutrophils with no FITC-labelled *S. aureus* = NEG control. (G) Killing of *S. aureus* by neutrophils during 90 minutes at 37°C in the presence of 5% heat-inactivated serum, compared by Mann Whitney test (H) Relative % of *S. aureus* killing was calculated by comparing % of killed *S. aureus* of neutrophil groups against the positive control (POS control). Relative % of *S. aureus* killing was compared between neutrophils pre-treated with anti-LILR or isotype mAb using a paired sample *t*-test ($n$ =7).







of *S. aureus*. Representative data is shown in Figure 5D Pre-treatment of neutrophils with anti-LILRB3 (relative % fluorescent neutrophils = 0.79, $p < 0.01$) and anti-LILRB4 (0.86, $p < 0.01$) induced significant reductions in the relative number of fluorescent neutrophils compared to isotype control mAb (1.06), indicative that LILR can modulate the ability of neutrophils to bind and/or phagocytose opsonized microbes (Figures 5D and E). Additionally, anti-LILRA6 (0.89, $p < 0.05$) also induced significant reductions in the relative number of fluorescent neutrophils compared to isotype control mAb, likely due to agonistic effects on LILRB3 in addition to LILRA6. To clarify that LILR modulate uptake of opsonized *S. aureus*, we measured the binding of fluorescently labeled IgG-opsonized *S. aureus* to neutrophils during 1 hr at 4°C. Under these conditions opsonized bacteria bind to, but are not phagocytosed, by neutrophils. Pre-treatment of neutrophils with anti-LILR mAb did not result in significant increases or decreases in the relative number of fluorescent neutrophils compared to the isotype control mAb (Figure 5E). This suggests neither anti-LILR mAb, nor LILR activation, block binding of *S. aureus* to neutrophils. This data suggests that LILR modulate phagocytosis, but not binding, of antibody-opsonized microbes.

Since both respiratory burst and phagocytosis are major antibacterial effector functions of neutrophils, this suggests that LILRs may modulate microbial killing. To test this hypothesis, we measured the ability of neutrophils to kill serum opsonized *S. aureus*. 60% of *S. aureus* were killed by neutrophils after incubation at 37°C for 90 minutes (Figure 5G). Pre-incubation of neutrophils with mAb against inhibitory LILRB3, but not LILRB4, increased the relative number of viable *S. aureus* compared to isotype control mAb, suggesting that LILR modulate microbial killing of neutrophils (Figure 5H). In contrast, mAb against activatory LILRs (LILRA1, A2 and A6), and isotype control, had no effect on microbial killing. There was enhanced variation in the data compared to ROS production or phagocytic uptake, which is likely to be reflective of the more complex biological process that was measured in these assays.

## Discussion

Neutrophils have a central role in innate and adaptive immunity as they detect and clear bacteria during infection, and have major roles in inflammation. Immunomodulatory receptors provide an important mechanism to regulate the activation threshold of immune cells, and there is increasing knowledge that immunomodulatory receptors have powerful roles in neutrophil biology. Here we provide robust evidence that LILRs are expressed on resting neutrophils, and demonstrate that they are potent immunomodulators of FcγR-mediated effector functions in neutrophils.

Expression, and ligand engagement, is required for immunomodulatory receptors to alter an immune activation threshold. Our data shows expression of LILRB3/A6 at the surface of resting neutrophils. Other immunomodulatory receptors such as CEACAM1, SIRL-1, SIGLEC5, SIRPα and CD200R are all consistently expressed at the neutrophil surface. The paired LILRB3/A6 form one of the major constituents of the secretory vesicle (SV) proteome (32), and as SVs are the most readily exocytosed organelle of a neutrophil it is likely that LILRB3/A6, and other receptors expressed within SVs, play important roles in early neutrophil functions such as extravasation and signaling. The high level of either or both LILRB3 and LILRA6 would suggest and important role for these LILR in modulation of neutrophil activation or in modulating activation thresholds. Accurate determination of the LILRB3:LILRA6 ratio on neutrophil surfaces, and the identification of their ligand(s), is required to take further steps to elucidating their functions. For other LILRs, we detected more variation in, and at times almost undetectable, expression. LILRB1 and LILRB2 have been demonstrated to be expressed on resting neutrophils in an earlier study (21). However, in our study we did not detect comparable levels of binding of anti-LILRB1 or anti-LILRB2 mAb to resting neutrophils to previous studies. Nonetheless, our data for LILRB2 is consistent with previous findings that surface expression increases following the stimulation of granule exocytosis (11), and was found predominately in the gelatinase & ficolin granule subset (32). However, enhanced surface expression of other LILR was not detected following exocytosis, suggesting that these LILRs are not expressed in granules or vesicles, or that expression is under the limit of detection in our assays.

Respiratory burst is a major microbial killing mechanism of neutrophils. Engagement of inhibitory receptors with agonists can suppress ROS production by neutrophils (5, 11, 18, 33, 34, 35). The activation of LILRB2 has been shown to have a potent ability to inhibit ROS production induced through FcγRs (11). We did not include anti-LILRB2 treatments in our assays because we could not detect binding of this mAb to resting neutrophils. We also found that LILRB3 and LILRB4 suppressed ROS production, but that at higher activation signals these LILR have no immunomodulatory effect. In order to draw comparison between the immunomodulatory functions of individual LILRs, comparative levels of receptor activation are required. As individual mAb possess different affinities and avidities for their target, they each mimic ligand engagement to different degrees. Thus, it is difficult to draw direct comparisons on receptor function in functional assays employing multiple mAb. Consequently, it is unclear which inhibitory LILRs have more pronounced modulatory effects. We did not identify convincing immunomodulatory effects from activatory LILR. Anti-LILRA6 activates LILRA6 and also LILRB3, and this may explain the absence of enhanced neutrophil functions.







Additionally, anti-LILRA1 was found to be an effective agonist on LILRA1CD3ζ reporter cells, but the level of activation was weaker than for other anti-LILR mAb. Therefore, it remains unclear if LILRA1 does not modulate neutrophil functions, or whether anti-LILRA1 was not a sufficiently strong LILRA1 agonist. In addition, we were unable to produce an effective LILRA2 2B4T reporter cell line, and therefore could not determine whether the anti-LILRA2 mAb used in this study had agonistic properties. The development of better LILR-specific agonists is required to further elucidate their ability to modulate FcγR stimulation of neutrophils.

Our data also demonstrate that LILRs modulate the ability of neutrophils to induce effector functions upon stimulation with Ig-opsonized *S. aureus*. Notably, activation of LILRB3 impaired ROS production induced through IgG1-opsonised *S. aureus*. This impairment was not detected for LILRB4 despite its ability to effect ROS production upon cross-linking of FcγRs. Further, it is unclear why anti-LILRB3 and anti-LILRA6 mAbs do not induce similar modulatory effects in this assay. The activation of inhibitory LILRs also impaired phagocytosis and killing of *S. aureus*, demonstrating that LILR activation can modulate major effector mechanisms and antimicrobial responses of neutrophils. There was variation in the ability of anti-LILRB mAb to suppress *S. aureus* killing than to suppress ROS production or phagocytosis, most likely because these experiments a more complex biological process than neutrophil activation and phagocytic uptake of bacteria. Interestingly, the data suggests that different LILRs impaired different functions. Activation of LILRB4 impaired *S. aureus* phagocytosis only. In contrast, activation of LILRB3 impaired both *S. aureus* phagocytosis and killing. In comparison, SIRL impairs ROS production induced through cross-linked FcR, but not opsonized *S. aureus*. Further, SIRL did not regulate phagocytosis of *S. aureus*, but did modulate killing through neutrophil extracellular traps (NETs) (35). Collectively, these findings indicate that inhibitory receptors differentially regulate FcγR mediated antimicrobial effector functions of neutrophils.

Emerging evidence indicates that some bacteria directly interact with LILRs. Specifically, *Staphylococcus aureus*, *Escherichia coli* and *Helicobacter pylori* bind LILRB1 and LILRB3 (36), whilst *Mycobacterium spp* recognizes LILRA1 and LILRB5 (28). As these bacteria are both commensals and opportunistic pathogens, and the bacterial ligands for these interactions largely remain to be elucidated, it is unclear whether LILRs have evolved as pathogen receptors or whether bacteria have evolved to exploit LILRs for modulating immune responses to benefit their survival. In either case, engagement of LILRs by bacteria could have profound impacts on neutrophil responses through modulating FcγR- and TLR-mediated functions.

Knowledge of the physiological roles of receptors requires accurate knowledge of their ligands. Both LILRB1 and LILRB2 bind to a broad range of classical and non-

classical MHC class I molecules. In particular, they have a high-affinity for HLA-G, an immune checkpoint molecule, suggesting that these LILR may help prevent neutrophils from becoming activated in the wrong places, such as the maternal-fetal interface (11, 37). However, there are few LILR ligands currently characterized with direct relevance to neutrophil biology. LILRA2 recognize microbially cleaved immunoglobulins (13), whilst LILRB1, LILRB2 and LILRB3 are reported to bind complement components (38). Identification of further characterization of native ligands will provide new tools to characterize the physiological role of LILRs in neutrophil biology.

In summary, we have characterized expression of LILRs on neutrophils, and demonstrated that inhibitory LILRs can suppress FcγR mediated activation and major effector functions of neutrophils. Inhibitory LILRs may provide an important checkpoint to control neutrophil inflammation, and an appropriate target for immunomodulatory therapeutics. To further resolve the roles of individual LILRs in neutrophil biology, characterization of biological ligands that are relevant to innate immunity is required.

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### Author contributions

AJM, KVK & JvS designed experiments. CDH and PGA cloned, expressed and purified mAb. AJM, YZ, KVK and EVW performed experiments and analyzed data. All authors were involved in interpretation of results. AJM & YZ drafted the manuscript, and all other authors revised the manuscript. All authors agreed with publication of the manuscript.

### Conflict of Interests Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.







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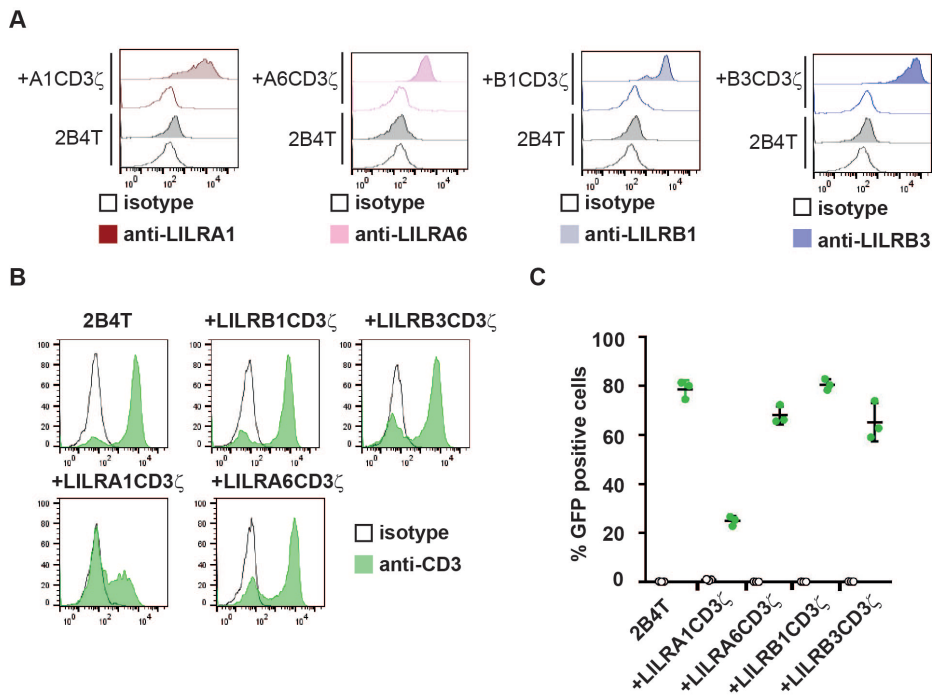
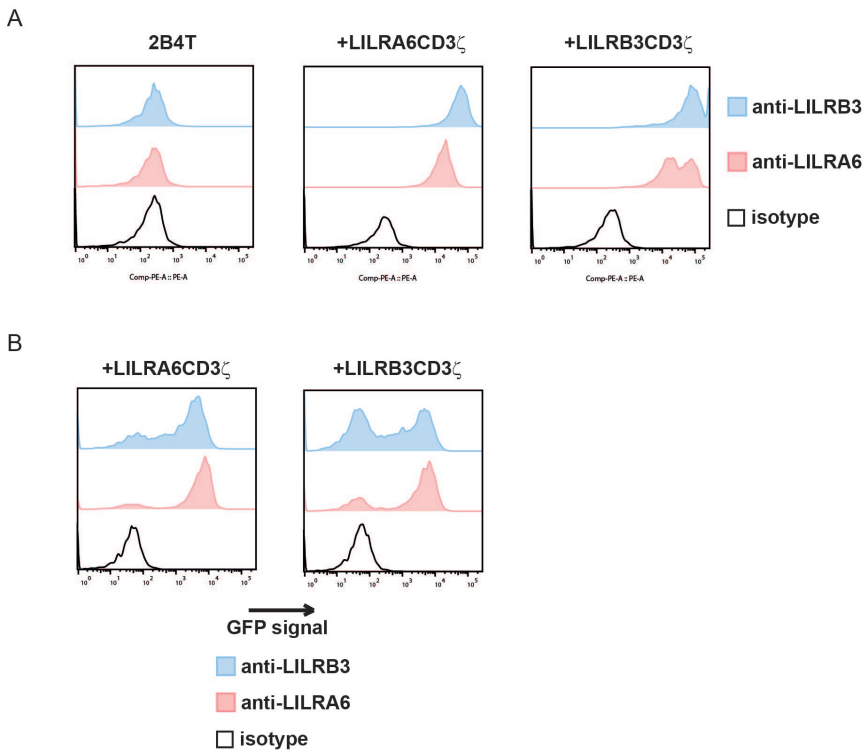



**Supplementary Figure 1. Expression of LILRCD3z in 2B4T cells.** (A) Binding of anti-LILR mAb or isotype control on the surface of empty 2B4T cell lines (2B4T) or those expressing LILRCD3ζ fusions is shown. Cell lines were incubated with 5 μg/ml of anti-LILR or isotype control mAb, and subsequently with anti-IgG-PE. Fluorescence of 2B4T cells was measured by flow cytometry. (B) All 2B4T cell lines (empty control cell, and those expressing LILRCD3ζ fusions) expressed GFP upon incubation with anti-CD3 mAb, but not isotype mAb. Representative plots are shown. (C) A paired sample *t*-test was used to compare % of GFP positive 2B4T cells after exposure to anti-CD3 (green) or isotype control (white) mAb ($n$ =3).

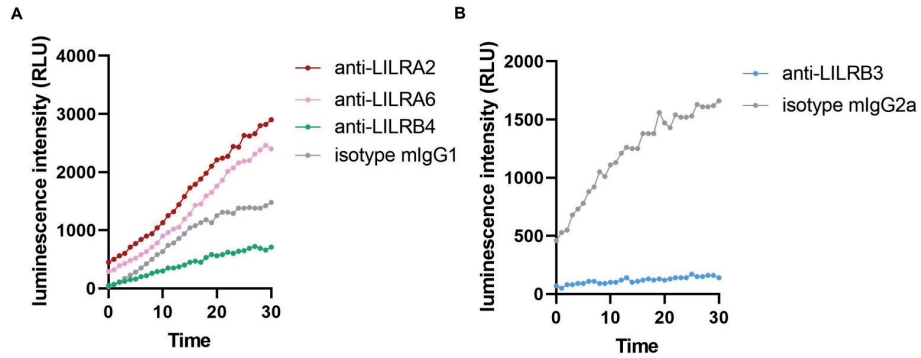



**Supplementary Figure 2. Agonistic effects of anti-LILRA6 and anti-LILRB3 mAb.** (A) Binding of anti-LILRA6 or anti-LILRB3 mAb or isotype control on the surface of empty 2B4T cell lines (2B4T) or those expressing LILRCD3ζ fusions is shown. Cell lines were incubated with 5 µg/ml of anti-LILR or isotype control mAb, and subsequently with anti-IgG-PE. Fluorescence of 2B4T cells was measured by flow cytometry. (B) GFP-expression in 2B4T cells expressing LILRCD3ζ (A6 or B3) fusion proteins or control 2B4T cells upon incubation in wells coated with anti-LILRA6, anti-LILRB3 or isotype mAb for 18 hours. Representative plots are shown. Fluorescence of 2B4T cells was measured by flow cytometry.










**Supplementary Figure 3. LILR modulation of FcγR-mediated ROS production.** Reactive oxygen species (ROS) production was stimulated with an HSA immune complex, formed with 4 µg/ml rabbit aHSA IgG. Neutrophils were pre-incubated with A) anti-LILRA2, anti-LILRA6, anti-LILRB4 or mouse IgG1 isotype mAb or B) anti-LILRB3 or mouse IgG2a isotype mAb.

CHAPTER 3

# Staphylococcal superantigen-like protein 13 activates neutrophils via Formyl Peptide Receptor 2

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## Abstract

Staphylococcal Superantigen-Like (SSL) proteins, one of the major virulence factor families produced by *Staphylococcus aureus,* were previously demonstrated to be immune evasion molecules that interfere with a variety of innate immune defenses. However, in contrast to characterized SSLs, that inhibit immune functions, we show that SSL13 is a strong activator of neutrophils via the formyl-peptide receptor 2 (FPR2). Moreover, our data show that SSL13 acts as a chemoattractant, induces degranulation and oxidative burst in neutrophils. As with many other staphylococcal immune evasion proteins, SSL13 shows a high degree of human specificity. SSL13 is not able to efficiently activate mouse neutrophils, hampering *in vivo* experiments.

In conclusion, SSL13 is a neutrophil chemoattractant and activator that acts via FPR2. Therefore, SSL13 is a unique SSL member that does not belong to the immune evasion class, but is a pathogen alarming molecule. Our study provides a new concept of SSLs; SSLs not only inhibit host immune processes but also recruit human neutrophils to the site of infection. This new insight allows us to better understand complex interactions between host and *S.aureus* pathological processes.








## Introduction

The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) is an opportunistic human pathogen that causes a wide range of diseases from mild skin infections to more serious life-threatening wound and systemic infections (1). In order to successfully invade and colonize the human host, *S. aureus* secretes a large arsenal of immune evasion molecules that specifically target components of the human innate and adaptive immune systems (2–4). These secreted proteins interfere with a range of immune defenses, which can be grouped into four categories: blocking, degradation, cell lysis and modulation (5). Despite the functional differences and diversity in targets, the staphylococcal immune evasion proteins are secreted proteins that show remarkable resemblances. These proteins contain very conserved structural properties (6). They are often small, varying in size between 8 and 35-kDa and have extreme isoelectric points (above 9 or below 5). Another common property of these proteins is that they are located on genomic clusters with other virulence factors. The secretome of *S. aureus* is predicted to contain up to 270 proteins, of which over 35 staphylococcal evasion molecules have been described (5). Identification and characterization of these secreted proteins will lead to a better understanding of the *S. aureus* pathological processes.

Neutrophils play a crucial role in protecting the host from *S. aureus* infections (7). Inherited or acquired neutrophil dysfunction, such as leukocyte adhesion deficiency and chronic granulomatous disease, lead to an increased risk of severe *S. aureus* infections (8). Disruption of physical barriers and invasion of *S. aureus* initiates the release of pro-inflammatory signals that promote neutrophil adherence to the vascular endothelium, extravasation and migration from the bloodstream towards to the site of infection (1). However, *S. aureus* can subvert neutrophil functions via the secretion of proteins that inhibit neutrophil recruitment and activation (9, 10). A variety of immune evasion proteins have been identified that specifically target neutrophil surface receptors. Some immune evasion proteins inhibit pro-inflammatory receptors such as Chemotaxis Inhibitory Protein of *S. aureus* (CHIPS) (11), Formyl Peptide receptor-like1 inhibitory protein (FLIPr), and the homologue FLIPr-like (FLIPrL) (12, 13). Other immune evasion proteins serve as toxins that use surface receptors to specifically lyse leukocytes, such as the bi-component toxins (PVL, LukAB, LukED) (14–16) and phenol soluble modulins (PSMs) (17). In addition, the PSMs act as potent neutrophil chemoattractants via human formyl-peptide receptor (FPR2) (18, 19). The FPR2 is a G protein-coupled receptor (GPCR) and a member of the formyl-peptide receptor family together with the archetype FPR1. Both receptors are present on neutrophils and myeloid cells and are considered as sensors for microbe associated molecular patterns (MAMPs). In contrast to the

FPR1, the FPR2 is a promiscuous receptor with various unrelated ligands, which include peptides, parts of proteins, lipids and small molecules, resulting in different intracellular responses in a ligand-specific manner (20).

Another group of secreted proteins, of which many are involved in immune evasion, are the Staphylococcal superantigen-like proteins (SSLs) (3). SSLs are a family of 14 proteins with structural similarity to Staphylococcal superantigens but lack the functional T-cell receptor binding domain and therefore exhibit no superantigenic activity (21). Moreover, structurally, the C-terminal β-grasp domain of these SSL proteins show homology to other staphylococcal immune evasion proteins like CHIPS. SSL1 to SSL11 are encoded on staphylococcal pathogenicity island 2 whereas SSL12, SSL13 and SSL14 are found on the immune evasion cluster 2 (IEC-2) (6, 22). The SSL gene cluster is expressed in all human and animal isolates of *S. aureus* examined to date, indicating that it is very stable and evolutionary important cluster for the organism (22–24). Furthermore, antibodies against the SSLs are detected in human serum, indicating that they are expressed *in vivo* and may play a role during infection (24, 25). Even though the SSLs are highly conserved and involved in innate immune evasion, they have distinct functions (21). It was reported previously that several SSL members located on the main cluster (SSL3, SSL5, SSL6, SSL7 and SSL10) are involved in inhibition of host immune responses (26–29). SSL3 and SSL4 have been described as Toll-like receptor 2 (TLR2) inhibitors and prevent neutrophil activation (30, 31). SSL5 interacts with neutrophil surface receptor CD162 and reduces neutrophil migration (9, 27). SSL6 was identified to interact with CD47 by screening a *S. aureus* secretome phage display library for binding to isolated human neutrophils (32). SSL7 binds to complement C5 and therefore prevents C5a production (33). In addition, SSL7 and SSL10 are associated with blocking complement activation by targeting IgA and IgG respectively (33, 34) . In contrast, none of the SSLs on the minor cluster (SSL12-14) have been functionally characterized.

In this study, we set out to identify new *S. aureus* proteins that interact with human neutrophils using a *S. aureus* secretome phage display library. In combination with Whole Genome Sequencing (WGS), SSL13 was identified to bind human neutrophils. We show that binding to human neutrophils is FPR2 dependent. Through this interaction, SSL13 activates neutrophils and acts as a chemoattractant. Furthermore, SSL13 induced an oxidative burst and degranulation in neutrophils. In contrast to many other immune evasion proteins that inhibit immune responses, we identified SSL13 as a chemoattractant and a neutrophil activator that acts via the FPR2.







## Experimental procedures

### Ethics statement

Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht ((METC-protocol 07-125/C approved March 01, 2010; Utrecht, The Netherlands). The use of animals was approved by the National Ethical Committee for Animal Experiments and performed according to the guidelines of the Central Animal Facility of the Utrecht University (Permit AVD115002016565).

### Reagents and antibodies

Monoclonal antibody (mAb) anti-His Tag (clone AD1.1.10, FITC-labeled) was purchased from LS Biosciences, and anti-CD62L (clone Dreg-56, FITC-labeled) and anti-CD11b (clone ICRF44, APC-labeled) were purchased from BD. The peptide MMK-1 (H-LESIFRSLLFRVM-OH) was synthesized by Sigma, and WKYMVM was synthesized by Bachem AG (Switzerland). WRWWWW-NH2 (WRW4) and Pertussis toxin were purchased from Tocris. Formyl-methionyl-leucyl phenylalanine (fMLP), TNF-α and cytochalasin B were from Sigma-Aldrich. Fluo-3-AM (acetoxymethyl ester) and Calcein-AM were purchased from Thermo Fisher.

### Cloning, expression, and purification of recombinant proteins

FLIPr, FLIPr-Like and N-terminal His-tag labeled SSL13 (His-SSL13) were cloned, expressed and purified as described (13, 35). For SSL13, primers were designed without signal peptide according to the published sequence of the gene NWMN_1076 for cloning into modified N-His6-TEV-(g)-pRSET vector (35). SSL13 was amplified from genomic DNA of *S. aureus* subsp. *aureus* strain Newman using the following primers: 5′- CGGGATCCCAATTTCCTAATACACCTATC-3′ and 5′-ATATGCGGCCGCTTAGTTTGATTTTTCGAG -3′. Restriction enzyme recognition sites are underlined. Recombinant protein was generated in *E.coli* Rosetta Gami(DE3) plysS by induction with 1 mM Isopropyl β-D-1-thiogalactopyranoside (Roche). His-tagged protein was isolated under native purification conditions using a 5ml HiTrap chelating HP column (GE Healthcare) with an imidazole gradient (10–250 mM; Sigma- Aldrich). The purified protein was analyzed on a 12.5% SDS- PAGE gel and showed one band corresponding to a mass of 26.8 kD (Fig. S1). For direct fluorescent labeling, His-SSL13 was mixed with 0.1 mg/ml FITC (Sigma-Aldrich) in 0.1 M carbonate buffer (pH 9.5) for 1 h at 4°C and subsequently separated from free FITC by overnight dialysis against PBS.

## Cells

Human leukocytes were isolated from human heparinized blood as described (32) and suspended in RPMI-1640 supplemented with 20 mM Hepes (Gibco) containing 0.05% HSA (Sanquin). HL-60 cells were purchased from ATCC, HL-60 cells stable transfected with the human-FPR2 (HL-60/FPR2), were kindly provided by F. Boulay (Laboratoire Biochimie et Biophysique des Systemes Integres, Grenoble, France). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FCS), 100 µg/ml streptomycin, 100 units/ml penicillin.

## Phage library construction and phage production

A *S. aureus* secretome phage display library was created as described earlier (32). Briefly, genomic DNA from *S. aureus* strain Newman was mechanically fragmented and fragments were cloned into the pDJ01 secretome phagemid vector (32) and transformed into TG1 *E. coli*. Phages lacking an active pIII protein were produced overnight by co-infection with Hyperphage® helper phages (Progen) at a multiplicity of infection of 10. Phages were purified and concentrated using PEG precipitation and resuspended in PBS to yield a final concentration of $2 \times 10^{11}$ phages/ml.

## Phage selection on isolated human neutrophils

1 ml of phage library was mixed with 1 ml isolated human neutrophils ($1 \times 10^7$ cells in RPMI-1640+0.05%HSA) and incubated on ice with gentle shaking for 30 min. Cells were washed twice by adding 50 ml cold buffer and centrifugation. Phages were eluted using 500 µl glycine 0.05M, pH2 for 5 min after which 62.5 µl neutralization buffer (2 M Tris-HCL pH 8.4) was added. Cells and cell debris were removed by centrifugation and phages were precipitated using 200 µl of 20% PEG/2.5 M NaCl for 30 min at room temperature. Sample was centrifuged at 14.000 rpm in an eppendorf centrifuge for 10 min at 4°C and supernatant was discarded. The pellet was suspended in 100 µl iodide buffer (10mM Tris-HCL, 1mM EDTA, 4M NaI, pH8) to disrupt the phage coat proteins and release the DNA. DNA was precipitated by adding 250 µl of 100% ethanol and incubated for 30 min at room temperature. Sample was centrifuged at 14.000 rpm in an eppendorf centrifuge for 10 min at 4°C after which the supernatant was discarded and the pellet containing the single stranded phage DNA was washed with 70% ice cold ethanol and dried to the air. The non-selected phage library was taken as a control.

## Phage library sequencing

Since the phage library was created using a pIII deficient helper phage, it consists of non-infectious phage particles. Therefore traditional phage selection with multiple rounds of selection and amplification is not possible and the library was







analyzed by genome sequencing using the Illumina MiSeq System. In order to add the MiSeq adapters to the isolated phage DNA, a PCR reaction was performed on the precipitated DNA using Phusion® HF polymerase (New England Biolabs), according to the manufacturer's recommendations. The primers were designed for compatibility with the Illumina MiSeq v2 sequencing kit. (Table S1 for primer sequences). The PCR product was purified using gel purification on an Ultra-pure 2% agarose gel and the purified DNA was quantified on a Qubit 4 fluorometer (Thermo Fischer Scientific). The purified sample was run on a 1% agarose gel to determine purity and determine mean fragment size.

Sequencing was performed by loading 3pM of the library onto a MiSeq v2 2x250bp sequencing kit and ran on an Illumina MiSeq System according to manufacturer's instructions. Sequence data was deposited in ENA under study accession number: PRJEB26168.

## His-SSL13 binding assay

To determine the binding of His-SSL13 to human leukocytes, a mixture of isolated neutrophils and mononuclear cells at $5 \times 10^6$ cells/ml was incubated with increasing concentrations of His-SSL13 for 30 min at 4°C while gently shaking. Cells were washed and incubated with FITC-labeled anti-Histag mAb while shaking. Cells were washed and resuspended in buffer containing 1% paraformaldehyde (PFA). The fluorescence was measured on a FACSVerse flow cytometer, and the different leukocyte populations (neutrophils, monocytes and lymphocytes) were identified based on forward and sideward scatter parameters.

To determine the binding of His-SSL13 to HL-60 cells, $5x10^6$ cells/ml were incubated with FITC-labeled SSL13 (SSL13-FITC) for 30 min at 4°C while shaking. Cells were washed and resuspended in buffer with 1% PFA. The fluorescence was measured by flow cytometry, and cell populations were identified based on forward and sideward scatter parameters excluding debris and dead cells.

## CD11b and CD62L expression on neutrophils

Neutrophils ($5 \times 10^6$ cells/ml) were incubated with different concentrations SSL13 for 30 min at 37°C. Subsequently, the cells were put on ice and incubated with anti-CD11b and anti-CD62L mAb for 45 min on ice. Cells were washed and fixed with 1% PFA in buffer. Expression of CD11b and CD62L was measured on a flow cytometer and data expressed relative to the buffer treated cells.

## Calcium flux in neutrophils and HL-60 cells

Calcium flux with isolated human neutrophils and HL-60 cells was performed in a flow cytometer as previously described (12). Briefly, cells at $5 \times 10^6$ cells/ml were

labeled with 0.5 µM Fluo-3-AM ester, washed and resuspended to a concentration of $1x10^6$ cells/ml. To measure cells continuously and be able to add stimulus without interruption in the FACSVerse flow cytometer, the Eppendorf tube adapter was used without tube while sampling cells from a 96-well plate on an elevated platform. Stimuli were added in a $1/10^{th}$ sample volume after a 10 seconds baseline recording and calcium flux monitored for 50 seconds post stimulation. Samples were analyzed after gating neutrophils, thereby excluding cell debris and background noises. Calcium flux was expressed as difference between baseline fluorescence (mean of time point 3 till 8 sec) and after addition of stimulus (mean of time point 20 till 60 sec).

### Chemotaxis

Neutrophil migration was measured in a 96-multiwell transmembrane system (ChemoTX; Neuro Probe) using an 8 µm pore size polycarbonate membrane(36). Cells were labeled with 2 µM calcein-AM for 20 min, and resuspended to a concentration of $2.5x10^6$ cells/ml in HBSS with 1% HSA. Wells were filled with 29 µl of chemoattractant, and the membrane holder was carefully assembled. Cells were pre-incubated with or without FLIPr and 25 µl was placed as a droplet on the membrane. After incubation for 30 min at 37°C in a humidified 5% $CO_2$ atmosphere, the membrane was washed extensively with PBS to wash away the non-migrating cells, and the fluorescence was measured in a fluorescence plate reader (CLARIOstar; BMG LABTECH) using 483 nm excitation and 530 emission filters. Percentage migration was calculated relative to wells containing the total fluorescence value of 25 µl cells.

### Myeloperoxidase (MPO) release

Neutrophils were treated for 10 min with cytochalasin-B and TNF-alpha with gently shacking, and without wash, subsequently incubated with buffer only, SSL13 or fMLP. Cells were centrifugated at 500 x g for 10 min and supernatant collected for MPO activity measurement (37). Therefore, 10 µl sample was mixed with O-Dianisidine substrate and $H_2O_2$ in phosphate buffer at pH 6.0 and measured continuously for 30 min at 37°C in a plate reader (FLUO star Omega) at 450 nm.

### Neutrophil oxidative burst assay

Horseradish peroxidase (HRP) and Isoluminol were used as a sensitive measure of the human neutrophil oxidative burst as described (38, 39). In white 96-well microtiter plates, a 150 µl reaction mixture of 6.25 x $10^4$ neutrophils per well in IMDM buffer with 0.1% HSA plus 50 µM isoluminol and 4 U/ml HRP was equilibrated for 5 min. Subsequently concentrated stimulus was added to activate the NADPH-oxidase







and emitted light immediately recorded continuously for 15 min in a Luminometer (Berthold) at 37°C. Data are expressed as relative light units (RLU).

### Mouse Experiments

In the mouse peritonitis model, 100 µg SSL13 in 0.5 ml PBS was injected into the peritoneum of 6- to 8-week-old female CD-1 mice. At 4 hours later, the mice were euthanized by cervical dislocation and abdominal cavities washed with two times 5 ml of RPMI medium containing 0.1% HSA and 5mM EDTA. In total 8 to 9 ml of peritoneal fluid was recovered and centrifuged at 1200 rpm for 10 min to collect the exudate cells. Cell pellets were resuspended in 500 µl buffer and counted with trypan blue in a TC20 automated cell counter (BioRad). Before immuno staining, cells were first preincubated with 100 µg/ml normal goat IgG for 15 min. We stained the samples with APC-conjugated antibody to mouse CD45 (leukocytes marker), PE-conjugated antibody to mouse Gr1 (neutrophil marker), and FITC-conjugated antibody to mouse F4/80(macrophage marker). Samples were analyzed on a flow cytometer.

Mouse neutrophils were isolated from bone marrow as described previously (40). Briefly, a bone marrow cell suspension was collected by flushing the femurs and tibias with 10 ml of cold HBSS + 15 mM EDTA + 30 mM Hepes + 0.1 % HSA. A two-layer Percoll density gradient (2 ml each in PBS) composed of 81% and 62.5% was used to enrich neutrophils from the total leucocyte population. Interphase between between 62.5% and 81% was collected. Cells were washed once with buffer and resuspended in PRMI1640 with 0.1% HSA.

Calcium fluxes in mouse neutrophils were determined as described above for human neutrophils with final concentrations of 10, 3, 1, 0.3, 0.1 and 0.03 nM of WKYMVM and 1000, 300, 100, 30, 10 and 3 nM of SSL13.

Mouse neutrophil binding assays were conducted essentially as described for human neutrophils.

## Results

### Phage library sequencing and identification of immune evasion proteins

To identify new *S. aureus* proteins that interact with human neutrophils using a *S. aureus* secretome phage display library. The sequencing run produced a total of 1,396 and 23,411 paired-end reads for the unselected and selection library, respectively. These reads were then quality-trimmed using nesoni clip v. 0.128 with the following parameters: --adaptor-clip yes --match 10 --max-errors 1 --clip-ambiguous yes --quality 10 --length 150 (http://www.vicbioinformatics.com/

software.nesoni.shtm). About 90% of the read pairs were retained and used for further analyses.

Quality-trimmed sequence reads were aligned to the Genbank database (accessed on July 20th, 2015) using BLAST+ 2.2.31. 3 sequences in the non-selected and 4 sequences in the selected library did not align with a *S. aureus* genome and were omitted from analysis. The read frequency was defined as the total count of identical reads. The total amount of unique sequences per annotated gene was defined as number of clones. The highest hit in the unselected library is annotated as a dUTPase with a read frequency of 14 all belonging to a single clone. The 96 reads with the highest read frequency after selection encode for 61 different proteins that are listed in table S2. There is a large increase in read frequency after selection. The highest read frequency with 883 reads encoding 7 unique sequences is annotated as a transmembrane protein involved in mannitol transport. The selection of transmembrane proteins when performing phage display selection on cells was also observed in earlier phage selections in our lab (data not shown). The presence of membranes appear to select for transmembrane domains especially transporter proteins like ABC-transporters. The second highest hit with 196 reads and 4 different clones identified the recently described *S. aureus* protein (SPIN) that binds neutrophil myeloperoxidase and promotes the intracellular survival of *S. aureus* after phagocytosis (35). Of the total of 61 identified proteins, 12 (20%) were described to play a role in host microbe interaction. Of these 11 were already functionally characterized and for 1 protein, SSL13, no known function has been described. The fact that SSL13 was identified in this selection suggests that it is involved in binding to neutrophils or its components.

## SSL13 specifically interacts with human neutrophils

To confirm that SSL13 interacts directly with human neutrophils, a three-fold dilution series of recombinant SSL13 with an N-terminal His tag was incubated with human leukocytes isolated from healthy donors. His-tagged SSL7 and SSL5 were included as negative and positive control neutrophil-binding proteins respectively (9, 41). We observed that SSL13 interacts with human neutrophils and monocytes in a dose dependent manner, but no significant binding was observed to lymphocytes (Fig. 1A-C).

Interestingly, binding experiments conducted at 37°C indicated that SSL13 activates neutrophils as shown by an increase in forward scatter compared with untreated cells (no protein) (42, 43) (Fig. S2 A-C). Activation of neutrophils generally alters the surface expression of major cell adhesion molecules, e.g. up-regulation of CD11b and down-regulation of CD62L (6). The effect of SSL13 on CD11b and CD62L expression was evaluated by flow cytometry. We observed that SSL13







enhanced the surface expression of CD11b and simultaneously down-regulated the expression of CD62L in a dose-dependent manner (Fig. 2A-B). In addition to the altered expression of surface adhesion molecules, activated neutrophils also exhibit intracellular release of calcium (44). We therefore measured the intracellular release of calcium after neutrophil exposure to a range of SSL13 concentrations (23–740 nM). In concordance with the cell receptor expression assay, our calcium flux data showed that SSL13 induces a transient dose-dependent release of $Ca^{2+}$ in neutrophils (Fig. 2C-D). Degradation of SSL13 by proteinase K completely abolished neutrophil activation indicating that the observed activation is not caused by a non-protein contaminant in the SSL13 preparation (Fig. S3 A-D). We used SSL7, that was expressed using the same protein expression system, as a control to rule out any specific activation by contaminant MAMPs like LPS or formylated peptides. To conclude, SSL13 specifically binds, and activates human neutrophils.

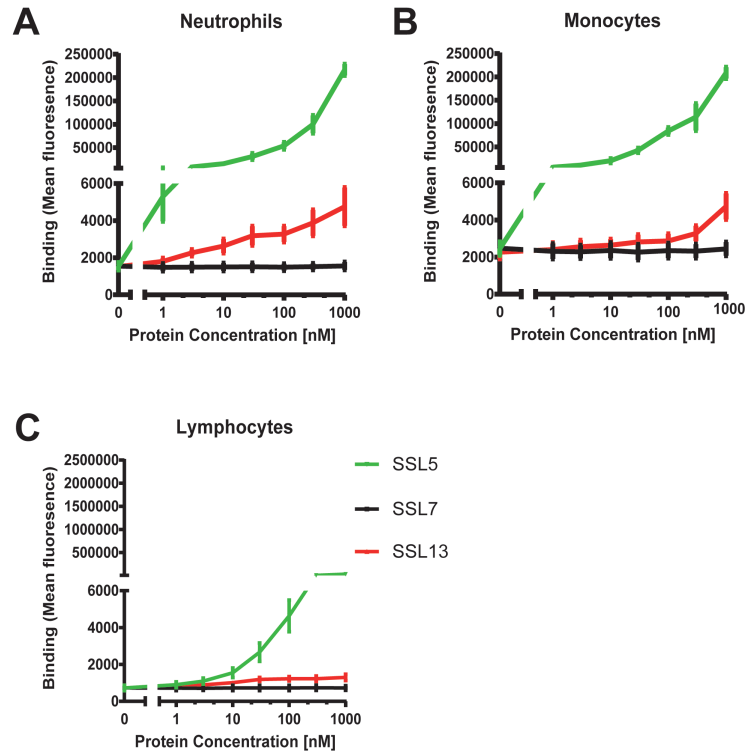


**Figure 1. SSL13 binds to human neutrophils, monocytes, but not lymphocytes.** Peripheral blood leukocytes were incubated with a three-fold dilution series of His-SSL13 for 30 min at 4 °C. Binding was detected with anti-His-FITC and analyzed by flow cytometry. The different cell populations were identified based on scatter parameters. His-SSL5 and His-SSL7 are positive and negative controls for binding respectively (A, B and C). Error bars are SEM of three biological replicates analyzed in duplicate.

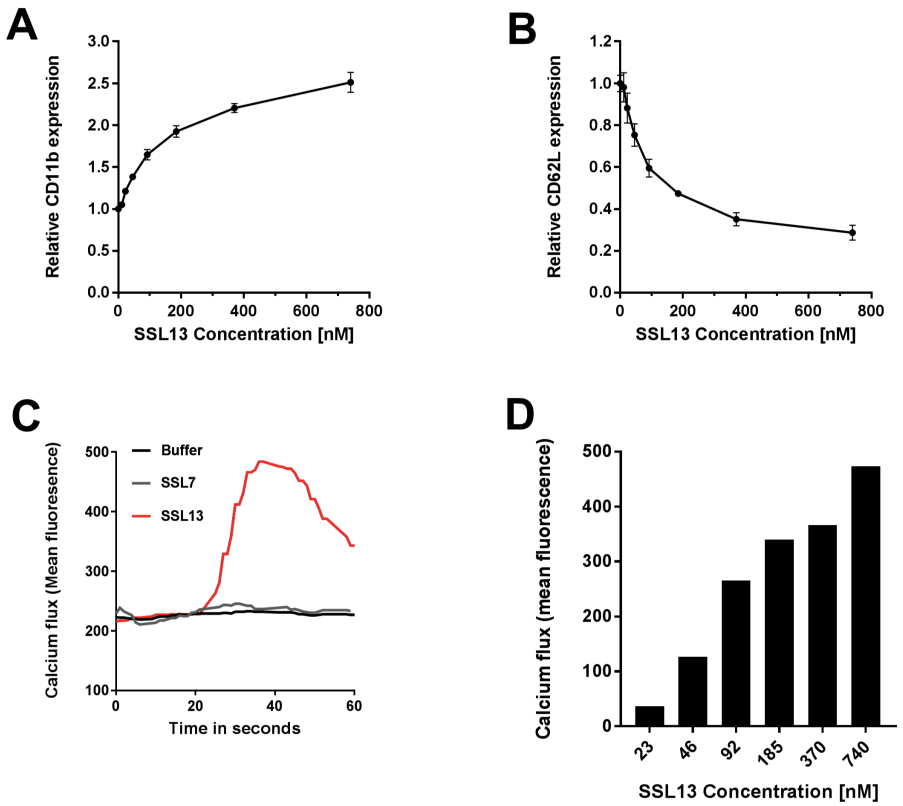


**Figure 2. SSL13 activates human neutrophils. Activation of isolated human neutrophils by increasing concentration of SSL13.** Increased CD11b expression (A) and decreased CD62L expression (B) are markers for neutrophil activation. Data are mean fluorescence ± SEM of three independent experiments. Addition of SSL13 induces cell activation measured as a transient release of intracellular calcium (C), in a concentration dependent manner (D). Data are from one representative experiment.

## SSL13 specifically binds and activates formyl peptide receptor 2

As SSL13 induced a rapid and transient release of intracellular $Ca^{2+}$, we examined whether SSL13 acts through a GPCR (Bestebroer *et al.*, 2010). Pertussis toxin (PTX) is a general antagonist of GPCR activation, and therefore blocks the release of intracellular $Ca^{2+}$ (46). For this purpose, neutrophils were preincubated with or without PTX for 1 h at 37°C with $CO_2$, and then stimulated with 370 nM SSL13 or fMLP as a reference PTX-sensitive stimulus (47, 48). Fig. 3A shows that PTX can block both SSL13 and fMLP induced neutrophil activation, which confirms that SSL13 utilizes a PTX-sensitive GPCR to induce this response.







To further investigate the responsible receptor, a set of well-characterized agonists and antagonists of neutrophil GPCRs were tested, including those for formyl peptide receptor 1 (FPR1) and 2 (FPR2), Leukotriene B4 receptor (BLTR1), platelet activating factor (PAF) receptor, Complement C5a receptor (C5aR), and the IL-8 receptors CXCR1 and CXCR2. We found that an FPR2 antagonist FLIPr inhibited SSL13 induced calcium mobilization, as well as SSL13 binding to human neutrophils (Fig. 3B-C). Although FLIPr also slightly inhibited the FPR1 activation, the control protein CHIPS, that specifically inhibits FPR1 (13), had no effect on SSL13-mediated neutrophil activation (Fig. 3C). Together, these experiments indicate that SSL13 elicits calcium fluxes in human neutrophils via FPR2.

To further confirm that FPR2 is the receptor for SSL13, we used HL60 cells stably transfected with or without human FPR2 (49, 50). Binding of FITC-labeled SSL13 was only observed for HL60/FPR2 and not for control HL60 cells (Fig. 3D). Furthermore, in order to evaluate the role of FPR2 in recognizing SSL13, we analyzed the intracellular $Ca^{2+}$ response to SSL13 of HL60 with or without FPR2. Fig. 3E shows that SSL13 induces a profound calcium flux in HL60/FPR2, but not in untransfected HL60 cells. The activation potential of SSL13 is comparable to the specific FPR2 agonistic peptide MMK-1 (Fig. 3E). Moreover, SSL13 activated the FPR2 transfected HL60 cells in a dose dependent manner (Fig. 3F). Finally, the induced calcium flux of the FPR2 transfected HL60 cells by SSL13 and MMK-1 can be inhibited by the FPR2-specific inhibitor FLIPr (Fig. 3G). These findings confirm that SSL13 specifically binds and activates cells via FPR2.

## SSL13 is involved in chemoattractant induced oxidative burst and degranulation of neutrophils

Triggering FPR2 induces many neutrophil effector functions, including chemotaxis, exocytosis and superoxide generation (51). To investigate whether SSL13 is a chemoattractant, neutrophil migration was measured in a 96-multiwell transmembrane system. Indeed, SSL13 stimulated chemotaxis of human neutrophils in a dose-dependent manner (Fig. 4A). Moreover, SSL13 induced chemotaxis in human neutrophils can be blocked by the FPR2 antagonist FLIPr (Fig. 4B).

To examine whether SSL13 is involved in FPR2-induced oxidative burst, a Reactive Oxygen Species (ROS) assay was performed. The peptides WKYMVM and MMK-1 can both induce FPR2-mediated ROS production, although WKYMVM is more potent and was therefore used as control in our experiment (52). Our data shows that SSL13 induced a modest oxidative burst compared with the control FPR2 specific peptide WKYMVM (Fig. 4C), but both SSL13- and WKYMVM-induced oxidative burst in human neutrophils could be blocked by FLIPr (Fig. 4C). Furthermore, we tested whether SSL13 could induce neutrophil degranulation by measuring myeloperoxidase (MPO)

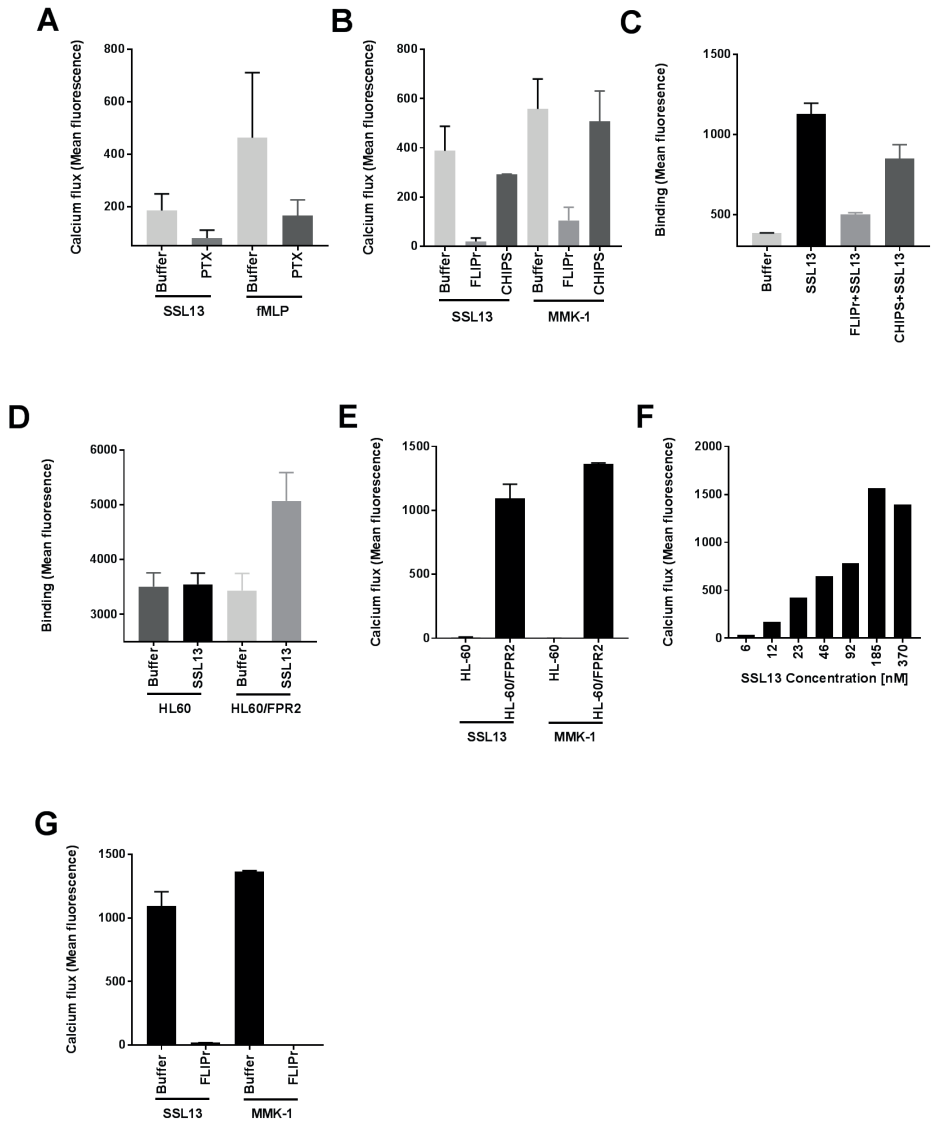


**Figure 3. SSL13 specifically binds and activates human Formyl Peptide Receptor 2.** (A) Neutrophils were preincubated with or without 3µg/ml PTX for 60min at 37 °C with $CO_2$ and then labeled with Fluo-3 AM. Neutrophil stimulation by SSL13 is sensitive to PTX. fMLP is the control ligand of FPR1, which is also sensitive to PTX. (B) Human neutrophil stimulation by SSL13 is inhibited by FLIPr, not CHIPS. (C) SSL13 specific binding to human neutrophils is blocked by FLIPr, not CHIPS. Data represent means ± SEM of three independent experiments. (D) SSL13 specifically binds to FPR2 transfected HL60 cells(HL60/FPR2), but not to control HL60 cells. (E) SSL13 induces profound calcium fluxes in HL60/FPR2 cells, but not in empty HL60 cells. MMK-1 is a synthetic control ligand of FPR2. (F) HL60/FPR2 cells stimulation by SSL13 is concentration dependent. (G) HL60/FPR2 cells stimulation by SSL13 is sensitive to FPR2-specific inhibitor FLIPr. Data are mean fluorescence ± SEM of three experiments.







activity in stimulated cell supernatant. MPO is one of the most abundant granule proteins in neutrophils and is efficiently released into the extracellular space during degranulation (53). Indeed, SSL13 induced neutrophil degranulation (Fig. 4D). Taken together, the functional outcomes of SSL13-induced neutrophil activation include chemotaxis, ROS production and neutrophil degranulation pointing toward a pro-inflammatory response of neutrophils to this staphylococcal protein.

To test whether SSL13 could act intracellular and is produced by *S.aureus* after uptake by human neutrophils, we generated a GFP promoter construct. Since SSL13 is part of an operon together with SSL12 and SSL14, the SSL12-13-14 promoter was cloned in front of GFP and transformed into *S.aureus Newman*. We did not observe expression of GFP under various standard culture conditions or after uptake of

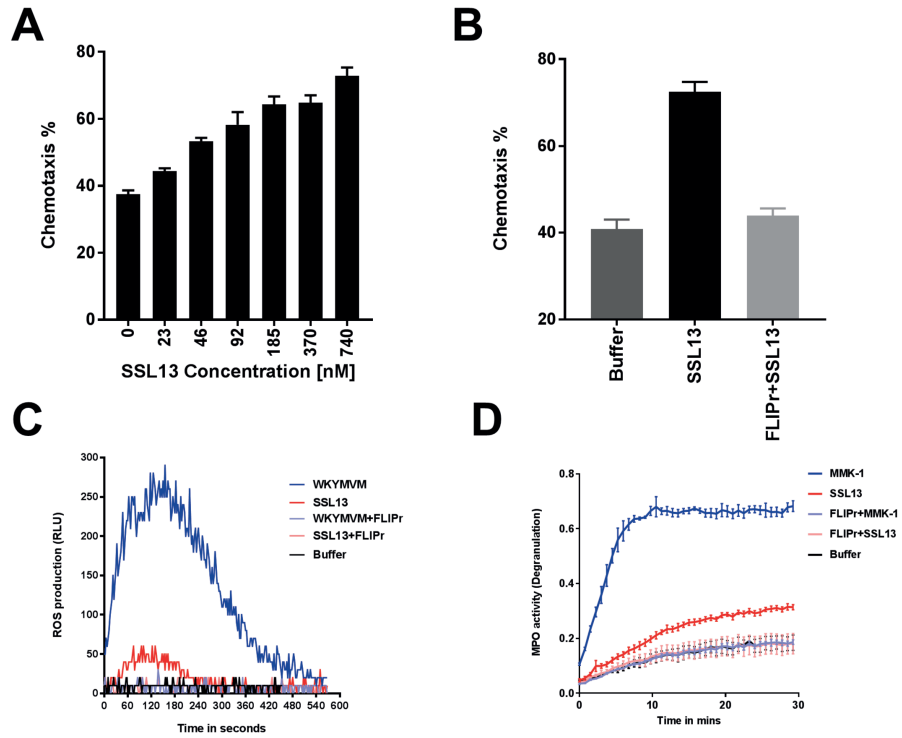


**Figure 4. SSL13 is involved in chemoattractant induced oxidative burst and degranulation of neutrophils.** (A) SSL13 stimulates chemotaxis in human neutrophils in a dose dependent manner. (B) SSL13-induced chemotaxis of human neutrophils is inhibited by the FPR2 antagonist FLIPr. (C) SSL13 stimulates FPR2 induced oxidative burst. WKYMVM is a synthetic control ligand of FPR2. (D) SSL13 modestly induces neutrophil degranulation via FPR2. MMK-1 is a positive control. (A and B) data represent means ± SEM of three experiments. (C and D) data from a representative experiment.

bacteria by phagocytes as seen with some other staphylococcal immune evasion proteins like SPIN (35) and PSMa (17) (data not shown here).

### SSL13 is not able to efficiently activate mouse neutrophils

Many other Staphylococcal immune evasion proteins show a high level of human specificity. In order to check the host-dependent activation of SSL13, we tested binding and activation of neutrophils isolated from mice bone marrow. SSL13 induced activation of murine neutrophils as shown by calcium mobilization. Treatment of murine neutrophils with WRW4, a known inhibitor of murine FPR2 (18), prevented SSL13-induced calcium flux. This indicates that neutrophil activation by SSL13 happened in a murine FPR2 dependent manner (Fig. 5A), although much higher concentrations are needed as compared to human neutrophil activation (Fig. 5B). In contrast, the specific FPR2 agonistic peptide WKYMVM showed similar activation ability to both human and murine neutrophils (Fig. 5C). However, we were unable to detect any SSL13 binding to murine neutrophils (data not shown).

Since there was a minimal but specific activation of mouse neutrophils, we tested whether SSL13 can provoke a neutrophil influx after injection of SSL13 into the mouse-abdominal cavity. We observed no increase in peritoneal neutrophil numbers at 4 h after intra-abdominal injection of 100 µg SSL13 (data not show). This indicates that SSL13 is highly adapted to specifically act on human neutrophils.

## Discussion

Previously, our group described a high-throughput binding selection strategy using phage display, to identify *S. aureus* immune evasion molecules. In this strategy, only secreted proteins of a bacterial genome are expressed on the surface of a filamentous phage, which is well suited to identify and characterize immune evasion proteins (32). Traditional phage selection strategies involve multiple rounds of selection and amplification and selecting single clones for sequencing and further analysis. Whole genome Illumina sequencing allows analysis of a phage library after only a single round of selection omitting library amplification that would undoubtedly lead to additional selection bias. Using this strategy we identified 12 proteins involved in host microbe interaction or immune evasion in a single round of selection indicating the enormous potential of this strategy. Furthermore, 8 conserved hypothetical proteins identified need further characterization, and may also be involved in the host microbe interaction. The identification of SSL13, a protein with previously unknown function, in this phage selection suggested an interaction between SSL13 and neutrophils. We show that SSL13 interacts specifically with FPR2, a member of







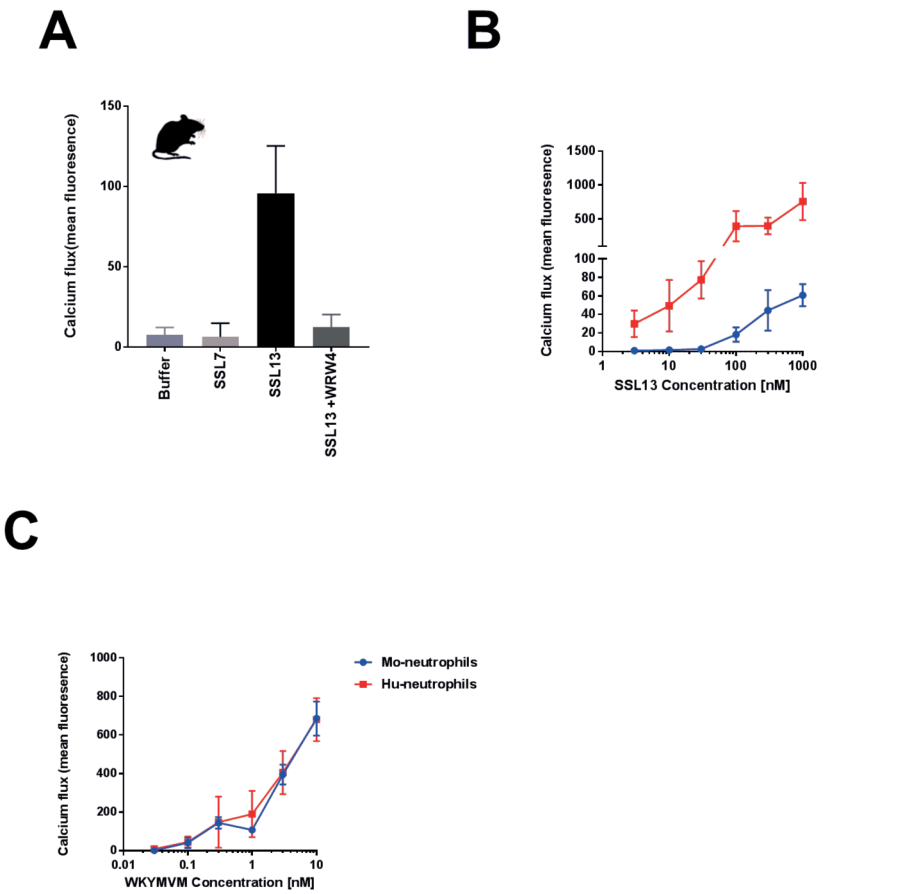


**Figure 5. SSL13 is not able to efficiently activate mouse neutrophils.** (A) SSL13 can induce activation of murine neutrophils, which can be inhibited by the mFPR2 antagonist WRW4. (B) SSL13-induced calcium fluxes in murine neutrophils are low compared to human neutrophils. (C) WKYMVM-induced calcium fluxes in murine neutrophils are similar to human neutrophils. Data are mean fluorescence ± SEM of three experiments.

the formyl-peptide GPCR receptor family involved in recognition of MAMPs. The FPR2 is expressed by human neutrophils and SSL13 interaction leads to neutrophil activation and chemotaxis.

The SSLs are a family of 14 secreted proteins which were previously demonstrated to modulate immune evasion (5, 6, 21). Genetic analyses of 88 clinical *S. aureus* strains revealed that the genes encoding SSL12, SSL13, and SSL14 are conserved among all strains (54). We also confirm that SSL13 is produced *in vivo* as antibodies can be detected in human serum (Fig. S4). Furthermore, in sharp contrast to the

SSLs located on SPI-2 that all have their own promoter, SSL12-13-14 share a single promotor. Our hypothesis is that SSL12-13-14 may be produced simultaneously by *S. aureus* under certain conditions and that their function could be linked. Unfortunately, we were unable to produce sufficient quantities of purified SSL12 and SSL14 to address this possibility. Just like the *S. aureus* bi-component toxin PVL requires LukS-PV and LukF-PV to properly lyse neutrophils (14), SSL12-13-14 might require the presence of all three proteins to elicit its maximum potential in immune modulation. Expression and secretion of most SSLs under standard culture conditions is very limited and only low amounts of protein can be found in the cell culture supernatant. There is an upregulation and expression of some SSLs under different stress conditions (55). We also did not observe SSL13 expression, using a GFP promotor reporter construct, under standard bacterial cell culture or after uptake by neutrophils.

SSL13 is not the only secreted molecule from *S. aureus* that is able to activate neutrophils PSMs, which are small peptides secreted by *S. aureus*, with a completely different structure compared to SSL13, are known to activate and attract both human and mouse neutrophils via FPR2 (17–19). In addition to this, micromolar concentrations of PSM have cell lytic activity which is independent from FPR2. Serum can fully block PSMs functions in both the cell lysis and FPR2-mediated neutrophil activation (17). However, SSL13 activity was not inhibited by serum and is not cytotoxic for neutrophils (Fig. S5). In contrast to PSMs, SSL13 showed a high degree of human specificity and was not able to efficiently activate mouse neutrophils. Another difference is the regulation and expression of PSMs, which was shown to be induced after uptake by neutrophils (17). So we believe this agonist is regulated differently from the PSMs, and thereby not a direct competitor for the same function via FPR2, but has its own yet unknown niche in the bacterial pathogenesis. Both PSMs and SSL13 require active synthesis and may be sensing by the MAMP receptor FPR2 is an adaption of our immune system. For PSMs, production inside the neutrophil to lytic concentrations could contribute to escape of *S. aureus* to enable survival. SSL13 could have a yet unknown additional function, may be in combination with the co-expressed SSL12 and SSL14.

FLIPr or its homologue FLIPr-like (FLIPrL) are located on the same IEC-2 cluster as SSL13, which are found in many, but not all, human *S. aureus* isolates (54). SSL13 is a neutrophil chemoattractant and activator that acts via FPR2, whereas FLIPr and FLIPrL bind and inhibit FPR2 signaling function (12, 13). This may contribute to the ability of *S. aureus* to adjust a favorable balance between neutrophil activation and inhibition. Similar to other staphylococcal immune evasion proteins, many of the SSL proteins harbor several distinct functions. Therefore, it is not unlikely that SSL13 may have another unique function beyond activating FPR2 signaling. To conclude,





SSL13 is a unique SSL member that does not belong to the immune evasion class, but is a pathogen alarming molecule acting on FPR2.

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## Supplement material

Degradation of SSL13 by proteinase K (Pro K). To check whether SSL13 was degraded by Proteinase K His-SSL13 was incubated with or without proteinase K for 30 min at 37 °C, The sample was added to 2 x SDS sample buffer and boiled for 10 min. 15µl sample was loaded onto a 12.5% SDS PAGE gel.

Serum antibody ELISA. ELISAs were performed as previously described. ELISA plate was coated overnight with 10 µg/ml of SCIN or SSL13 in PBS. Always three washes with PBS-0.05% Tween-20 before the next step. First, plate was blocked with 4% BSA in PBS-0.05% Tween-20 in 37 °C for 1h. Second, plate was incubated with a three-fold dilution series of healthy human pooled serum in 37 °C for 1h. Finally, peroxidase-conjugated goat α-human IgG (SouthernBiotech) was added at a 1/5000 dilution. Peroxydase activity was detected with 3,3′,5,5′-tetramethylbenzine (TMB) for 5-10 min and the reaction was terminated using $H_2SO_4$.

Cell permeability assay. Intracellular staining dye 4′,6-diamidino-2-phenylindole (DAPI) was used for cell permeability check. Briefly, human neutrophils were incubated with SSL13 or PSM in presence or absence 10% human pooled serum in a volume of 50µl in RPMI containing 0.05% human serum albumin for 30 min at 37 °C. Cells were analyzed by flow cytometry.

- His-SSL13 sequence

  MHHHHHHENLYFQGSQFPNTPINSSSEAKAYYINQNE
  TNVNELTKYYSQKYLTFSNSTLWQKDNGTIHATLLQF
  SWYSHIQVYGPESWGNINQLRNKSVDIFGIKDQETIDS
  FALSQETFTGGVTPAATSNDKHYKLNVTYKDKAETFT
  GGFPVYEGNKPVLTLKELDFRIRQTLIKSKKLYNNSY
  NKGQIKITGADNNYTIDLSKRLPSTDANRYVKKPQNA
  KIEVILEKSN
- Number of amino acids: 233
- Molecular weight: 26814.88
- Theoretical pI: 9.08
- Removed 1-23aa signal peptide

**Figure S1. Cloning, expression, and purification of SSL13.**

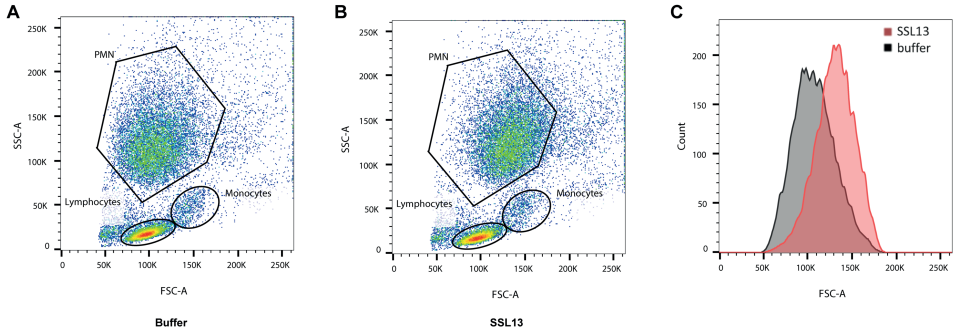



**Figure S2. SSL13 alters human neutrophils forward scatter.** Peripheral blood leukocytes were incubated with buffer or 370 nM His-SSL13 for 30 min at 37 °C. Cell scatters were detected and analyzed by flow cytometry. The different cell populations were identified based on scatter parameters (A and B). SSL13 increases neutrophils forward scatter compared with untreated cells (C). Data are from one representative experiment.

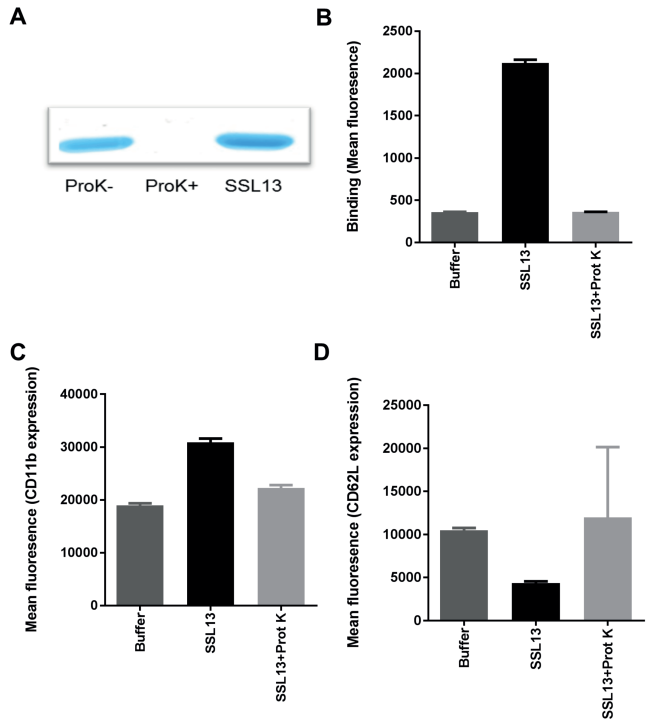



**Figure S3. SSL13 specifically interacts with human neutrophils.** His-SSL13 incubated with or without proteinase K for 30 min at 37 °C. (A) SSL13 is degraded by proteinase K. (B) Degradation of SSL13 by proteinase K completely abolished the neutrophil binding. Neutrophil activation was measured by CD11b (C) and CD62L expression (D) Cell activation by SSL13 is inhibited by pretreatment with proteinase K. Data are mean fluorescence ± SEM of three experiments.







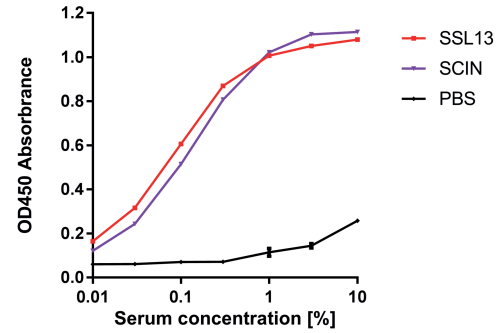
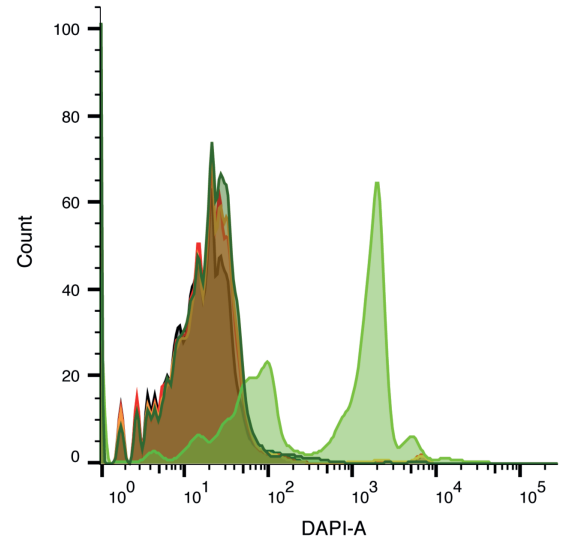



**Figure S4. Detection of SSL13 serum antibodies.** His-SSL13 and His-SCIN was coated to an ELISA plate overnight at 4 °C. After incubation with a three-fold dilution series of healthy human pooled serum, binding of human serum antibodies was detected using a goat-anti-human IgG-HRP antibody. His-SCIN, another secreted *S. aureus* protein, is a positive control. Data is from one representative experiment.




| | Sample Name | Subset Name | Geometric Mean : DAPI-A |
|---|---|---|---|
| | PSM+Serum | PMN | 11,7 |
| | PSM | PMN | 281 |
| | SSL13+Serum | PMN | 9,62 |
| | SSL13 | PMN | 9,06 |
| | Buffer | PMN | 8,21 |

**Figure S5. SSL13 has no cytotoxic activity.** Human neutrophils were incubated with PSM or SSL13 in presence or absence of serum. The membrane impermeable DNA stain DAPI was used to label dead cells. Phenol Soluble Modulins (PSM) are cytotoxic and this toxicity is inhibited by preincubation with serum. SSL13 is not cytotoxic as incubation with SSL13 does not lead to an increase in DAPI signal.

**Table S1. Primers for genome sequencing**

| | primers |
|---|---|
| pDJ01NextN701 | CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGGACAATCCTGAACGCAGAAATCAAGAGG |
| pDJ01NextN702 | CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCAGGACAATCCTGAACGCAGAAATCAAGAGG |
| pDJ01NextN501 | AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAAAATCACCGGAACCAGAGCCACCACCC |
| pDJ01NextN502 | AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCAAAATCACCGGAACCAGAGCCACCACCC |

**Table S2. Proteins with the highest read frequency after phage display selection**

| Identified Protein | read freq. | # of clones | Protein function |
|---|---|---|---|
| PTS mannitol transporter subunit IICB | 883 | 7 | |
| Peroxidase Inhibitor | 196 | 4 | Inhibits human neutrophil myeloperoxidase |
| NWMN_0791 conserved hypothetical protein (HlyC/CorC family transporter) | 109 | 4 | Conserved hypothetical protein |
| Immunoglobulin G-binding protein Sbi | 101 | 2 | Inhibits opsonophagocytosis |
| Iron ABC transporter | 79 | 2 | |
| Superantigen-like protein SSL4 | 72 | 7 | Binds sialated glycoproteins on myeloid cells |
| Acetyl-CoA acetyltransferase | 54 | 3 | |
| Methicillin resistance protein FmtB | 50 | 3 | |
| TIGR01440 family protein | 47 | 1 | Conserved hypothetical protein |
| Chemotaxis Inhibitory Protein | 41 | 2 | Inhibits C5aR and FPR1 mediated chemotaxis |
| Mannitol transporter protein | 39 | 1 | |
| Meticillin resistance cassette | 39 | 4 | |
| FPR2 inhibitory protein | 36 | 2 | Inhibits FPR2 on neutrophils and monocytes |
| Cell wall associated fibronectin-binding protein | 48 | 3 | Binds plasminogen and elastin |
| N-acetylmuramoyl-L-alanine amidase | 26 | 3 | |
| Ribosomal RNA methyltransferase FmrO domain protein | 25 | 1 | |
| CBS domain protein | 24 | 1 | |
| Dihydroorotase | 19 | 1 | |
| DUF4064 domain-containing protein | 19 | 1 | |
| ATP-dependent Clp protease, ATP-binding subunit ClpC | 16 | 1 | |
| Non-coding region between hypothetical proteins | 16 | 1 | |
| NWMN_0280 conserved hypothetical protein similar to ORF061 of Bacteriophage ROSA | 15 | 1 | Conserved hypothetical protein |
| Aldehyde dehydrogenase family protein | 14 | 1 | |
| Capsular polysaccharide biosynthesis protein CapJ | 14 | 1 | |
| Conserved hypothetical protein (downstream LukED) | 14 | 1 | Conserved hypothetical protein |
| NWMN_2283 hypothetical protein DUF4889 superfamily(upstream conserved hypot 2282) | 14 | 1 | |
| Fibronectin binding protein | 13 | 1 | Binds plasminogen and elastin |
| Intergenic region between BAF68719.1 en BAF68720.1 | 13 | 1 | |
| Phage anti-repressor for bacteriophage phiNM1 | 13 | 1 | |
| Branched-chain amino acid transporter II carrier protein | 12 | 1 | |
| Conserved hypothetical protein similar to 5′-nucleotidase family protein multifunctional 2′,3′-cyclic-nucleotide 2′-phosphodiesterase/5′-nucleotidase/3′-nucleotidase | 12 | 1 | Conserved hypothetical protein |
| Enterobactin ABC transporter permease | 12 | 1 | |
| LLM class flavin-dependent oxidoreductase | 12 | 1 | |
| Multidrug resistance transporter protein B | 12 | 1 | |
| NWMN_2018 conserved hypothetical protein | 12 | 1 | Conserved hypothetical protein |
| Staphyloxanthin biosynthesis protein | 12 | 1 | Inhibits oxygen radicals |
| Bi-component leukocidin LukGH subunit H | 11 | 1 | Leukotoxin |
| Clumping factor A | 11 | 1 | Binds fibrinogen |
| Cysteine protease staphopain B | 11 | 1 | Inactivates neutrophil elastase and complement proteins |
| NWMN_0338 conserved hypothetical protein | 11 | 1 | Conserved hypothetical protein |
| Monovalent cation/H+ antiporter subunit D | 10 | 1 | |
| Na+/alanine symporter family protein | 10 | 1 | |
| NWMN_0344 conserved hypothetical protein (ABC-2 transporter permease) | 10 | 1 | Conserved hypothetical protein |
| Bi-component gamma-hemolysin HlgAB subunit A | 9 | 1 | Leukotoxin |
| Iron-regulated heme-iron binding protein IsdB | 9 | 1 | |
| Lantibiotic leader peptide processing serine protease | 9 | 1 | |
| Minor structural protein for bacteriophage phiNM3 | 9 | 1 | |
| NWMN_0218 staphyloxanthin biosynthesis protein | 9 | 1 | |
| NWMN_1584 conserved hypothetical protein | 9 | 1 | Conserved hypothetical protein |
| Peptidase M23B | 9 | 1 | |
| Secretory antigen precursor SsaA homolog | 9 | 1 | |
| Superantigen-like protein SSL13 | 9 | 1 | Activates FPR2 on neutrophils and monocytes |
| 16S rRNA methyltransferase | 8 | 1 | |
| Manganese ABC transporter substrate-binding protein | 8 | 1 | |
| MarR family transcriptional regulator | 8 | 1 | |
| Multifunctional 2′,3′-cyclic-nucleotide 2′-phosphodiesterase/5′-nucleotidase/3′-nucleotidase | 8 | 1 | |
| rRNA-23S ribosomal RNA | 8 | 1 | |
| Truncated transposase for IS1272 | 8 | 1 | |
| Truncated triacylglycerol lipase precursor | 8 | 1 | |

**Table S2. Proteins with the highest read frequency after phage display selection.** A *Staphylococcus aureus* phage display library was selected for binding against isolated human neutrophils. The selected library was analyzed by whole genome sequencing. Table shows the top hits after selection based on read frequency and grouped by number of different clones. Function of previously characterized immune evasion proteins and hypothetical proteins are listed.

CHAPTER 4

# A novel immunoglobulin binding protein with repeating domains from intestinal bacterium

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*Manuscript in preparation*

## Abstract

The human body is a constantly changing ecosystem comprised of trillions of microbial organisms collectively known as the microbiome. The microbiome is essential in maintaining homeostasis and has a profound effect on the development and maturation of the immune system. The microbiome of the intestine comprises the largest variety of bacteria in the human body. Although the genetic composition of the intestinal microbiome is more clearly, only little is known about the specific mechanisms underlying host microbiome interactions, especially from a microbial perspective. A high throughput phage display selection strategy was performed to identify secreted intestinal bacterial proteins that interact with host immune components. Using this high throughput selection we identified a secreted multi-domain protein of *Ruminococcus*, which we termed ProteinR. Interestingly, we also discovered that ProteinR act as an immunoglobulin binder. ProteinR binds the Fab-domain of human antibodies (IgA, IgM and IgG) with high affinity and efficiently blocks Fc mediated phagocytosis *in vitro*. Furthermore, ProteinR forms complexes with immunoglobulins that induced neutrophil ROS production. Homology searches revealed similar proteins present in other *Ruminococcus* sp and *Blautia* sp, but they vary in domain organization. This may illustrates the importance of the presence of a gene rather than the presence of a certain species in the microbiome.






## Introduction

After our birth the intestinal tract becomes colonized with thousands of species of microorganisms, collectively referred to as the "microbiome" (1). The human gut microbiome has become a major research topic of many different groups over the recent years. The increasing number of publications on the microbiome makes it increasing clear how variations and changes in the composition of gut microbial ecology and gut microbial network connectivity contribute to the metabolic regulation of important pathways in the host (2–7). In addition, these studies help us to understand how the gut microbiome plays an instrumental role in our physiology, both in health and disease (2, 8–12). Particularly the $10^{14}$ bacteria that make up the gut microbiome are known to establish bi-directional and symbiotic relationship with human cells throughout our entire life (10, 13). Alterations in microbiome composition have been associated with many non-infectious diseases including inflammatory bowel conditions, autoimmune diseases, cancers and diabetes (14–16). To keep the gut symbiotic environment, the bacteria present within the gut have to produce proteins that ensure their survival within the host without endangering it. This requires a perfect balance between pro-inflammatory and regulatory responses that shape our immune system (10). In other words, the intestinal epithelium and associated immune system components must be able to both tolerate these beneficial microorganisms and mount a response to invading pathogens. While, this paradoxical function is crucial to overall gut health, little is known about the exact proteins that are produced by the microbiome that promote immune tolerance by interacting with the intestinal immune system or even interacting with the systemic immune system.

To identify potential immune-modulatory molecules from gut microbiota, a high throughput phage display selection strategy was performed to identify secreted intestinal bacterial proteins that interact with host immune components. We constructed a phage display library of the human intestinal microbiome by using feces from 9 healthy volunteers. In a phage selection on human complement component C3, a secreted hypothetical protein was found from multiple gut microorganisms, most belonging to different *Ruminococcus* species. *Ruminococcus* species are nonpathogenic intestinal Gram-positive anaerobic bacteria. Here, we renamed this protein as ProteinR. ProteinR has a molecular weight of approximately 62.7 kDa and is composed of a short N-terminal, five domains and a relatively long C-terminal tail. The first two domains are identical, while the other domains show a high similarity to first two repeats. ProteinR is able to bind several immunoglobulins (IgG, IgA and IgM) from different isotypes and subtypes derived from different organisms. Surprisingly, our data showed that ProteinR was able to inhibit Fc

mediated neutrophil phagocytosis. Furthermore, ProteinR immune complex formed with immunoglobulin induced neutrophil ROS production via Fc receptor.

In this study, we report the ProteinR, as the first immunoglobulin binding protein secreted by multiple gut microorganisms, was able to interact in vitro with a large repertoire of mammalian Igs, including secretory IgA. This remarkable observation may provide a new tool in the antibody purification study. But also which gave the impression that non-pathogenic gut bacteria modulate the gut immunity and homeostasis by secreting a group of immunoglobulin binding protein.

## Material and methods

### Ethics statement

Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht ((METC-protocol 07-125/C approved March 01, 2010; Utrecht, The Netherlands). Recombinant Protein L was obtained from Life Technologies.

### Reagents and antibodies

The information of all the Monoclonal (mAb), polyclonal immunoglobulins and conjugates to detect primary immunoglobulins used in this study are shown in Table S1, Table S2 and Table S3 respectively. Human recombinant mAbs variable region sequences were cloned into vectors containing the constant regions for kappa light chain and the appropriate Ig class heavy chains. Constructs were transfected into EXPI293F cells (Thermo Fisher) propagated in EXPI293 Expression medium and Ig isolated from the supernatant using HiTrap Protein A, Hi-Trap Protein-G, anti-Kappa, or CaptureSelect anti-IgM columns. IdeS protease derived from *Streptococcus pyogenes* was from Promega.

### Community DNA preparation from Fecal samples

Feces samples from nine healthy volunteers were collected and stored at -80°C until DNA isolation was performed. For DNA preparation, 250 µl Guanidine Thiocyanate, 40 µl 10% N-Lauroyl Sarcosine and 500µl 5% N-Lauroyl Sarcosine were added to each fecal sample (approximately 200 mg). Samples were vortexed and incubated at 70°C for 1h. Subsequently, 750 µl glass beads were added to the samples and vortexed after which a Bead-Beater was used to lyse the cells and a small volume Polyvinylpolypyrrolidone (PVPP) was used to extract impurities. Samples were centrifuged for 3 min at 14000 rpm. Supernatant was transferred to sterile 2 ml eppendorf tubes and 500 µl of TENP was added to the pellet and resuspended





followed by another 3 min centrifuge step. Supernatant was pooled with the previously collected supernatant. This step was repeated 3 times. The total collected supernatant was centrifuged for 3min at 15000 rpm and transferred to two sterile 2 ml Eppendorf tube after which 1 ml of isopropanol was added and incubated for 10 min at RT, mixed gently by inversion and subsequently centrifuged for 10 min at 15000 rpm. Supernatant was discarded and the pellet dried. 450 µl phosphate buffer and 50 µl of potassium acetate was added to one of the two tubes. After the pellet was completely resuspended, the total volume was transferred to the second tube pellet was resuspended and samples were incubated at 4 °C for 90 min. Samples were centrifuged for 30 min at 15000 rpm, 4 °C. Supernatant was collected and transferred to a fresh 2 ml eppendorf tube. The supernatant was incubated with 20 µg/ml of RNAse for 30 min at 37 °C. 50 µl of sodium acetate (3M, pH 5.2) and 1 ml of 100% ethanol was added and gently mixed by inversion. Samples were centrifuged for 5 min at 14000 rpm after which the ethanol layer was removed and the pellet was washed twice with 70% ethanol. Samples were dried under a laminar flow hood and once dry the pellets were dissolved in 200 µl Tris Elution buffer. DNA samples were analyzed on an agarose gel and confirmed to be pure.



### Phage library and selection

A human intestinal microbiome secretome phage display library was created as described earlier (17). Briefly, fecal DNA was mechanically fragmented and fragments were cloned into the pDJ01 secretome phagemid vector (18) and transformed into TG1 *E. coli* to yield a library with a complexity of $2.1 \times 10^9$ individual clones. Phages were produced overnight by coinfection with VCSM13 helper phages (Agilent technologies) at a multiplicity of infection of 10. Phages were purified and concentrated using Polyethylene glycol (PEG) precipitation and resuspended in PBS to yield a final concentration of $2 \times 10^{11}$ phages/ ml.

Phage selection was performed in an ELISA plate coated with a total amount of 5 µg target protein in PBS overnight at 4°C and blocked with bovine albumin. Three consecutive rounds of selection were preformed and 48 selected clones were amplified and sequenced using primers InsertF (5′-GGAAGAGCTGCAGCATGATGAAA-3′) and InsertR (5′-CACCGTAATCAGTAGCGACAGAA-3′).

### Cloning, expression, and purification of recombinant proteins

All Protein R constructs, were cloned, expressed and purified as described (19). In short, gBlock/primers were synthesized (Integrated DNA Technologies) according to the published sequence of the gene EES75741.2 hypothetical protein RSAG_03154 from *Ruminococcus sp.* 5_1_36BFAA for cloning into the modified pRSET-AAA-HHHHHH (only for D12, D123 and D1234) and pRSET-LPETG-AAA-HHHHHH (for the rest) vector

via Gibson assembly/PCR. Recombinant proteins were generated in *E.coli* Rosetta Gami (DE3) plysS by induction with 1 mM Isopropyl β-D-1-thiogalactopyranoside (Roche). His-tagged protein was isolated under native purification conditions using a 1ml HiTrap chelating HP column (GE Healthcare) with an imidazole gradient (10–250 mM; Sigma-Aldrich). Constructs that contain the LPETG motif were used for direct labeling with fluorescein or biotin using sortaging. Proteins were mixed with 5μM His-tagged-sortase 7+ (20) and 0.5 μM GGGK-FITC or GGGK-biotin in 50 mM Tris with 150 mM NaCl buffer (pH 7.5) for 2 h at 4°C. Subsequently the labeled proteins were purified over a 1ml HiTrap chelating HP column to eliminated non-sortagged proteins and sortase enzyme. Free FITC or Biotin was removed by overnight dialysis against 50 mM Tris with300 mM NaCl.

Protein-A single domain B as wt (Spa-B-wt), only Fab binding variant (Spa-B-KK; Q9K, Q10K) and only Fc binding variant (Spa-B-AA; D37A, D38A) were cloned into pRSET-LPETG-AAA-HHHHHH vector, further purified and labeled as mentioned above.

### Fragmentation of IgG with IdeS protease from Streptococcus

Monoclonal IgGs were cleaved into F(ab')2 and Fc- fragments by using IdeS protease. According to manufacturer instructions, 500 μg/ml IgG was incubated with 500 units of IdeS for 90 min at 37°C while shaking. Subsequently, the Fc and F(ab')2 fragments were separated by gel filtration chromatograph using a Superdex 300 column. Final products were evaluated on a 4-12% gradient SDS-PAGE gel and tested by ELISA.

### Enzyme-Linked Immuno Sorbent Assay (ELISA)

MaxiSorp plates (Nunc) were coated overnight at 4 °C with 50 μl of the different ProteinR constructs at 3 ug/ml in a 0.1M Sodium Carbonate buffer. Plates were washed 3 times with PBS-Tween (0,05% T), and blocked for one hour with 4% skimmed milk in PBS-T (at 37°C. After 3 washes, 50 μl of the tested immunoglobulins or their fragments were added to the wells with various concentrations using in PBS-T with 1% skimmed milk for 1 hour at 37°C. Plates were washed three times and incubated with the appropriate secondary horseradish peroxidase (HRP) conjugated immunoglobulins for 1 hour at 37°C. Plates were developed with TMB substrate reaction stopped with 1N sulfuric acid. The iMark™ Microplate Absorbance Reader was used to detect the color intensity of the plates at 450 nm.

### Binding and competition on mAmetrine-labeled *S. aureus* Newman-spa/sbi-KO

To determine the influence of ProteinR on the antigen recognition by the anti-WTA antibodies, a flow cytometer assay was developed with *S. aureus* Newman- spa/sbi–KO





with a constitutively and robustly expressed mAmetrine (21). Bacteria were incubated with 1 μg/ml anti-WTA antibodies for 30 min at 4°C while shaking at 750 rpm. After washing, bacteria were mixed with various concentration of different ProteinR constructs for another 30 min. Binding of the different ProteinR constructs was determined with goat-anti 6x His tag for 30 min followed by PE conjugated Donkey anti goat IgG.

To check whether ProteinR interfered with anti-WTA binding epitope, a fixed concentration of anti-WTA mAb was pre-incubated with the different ProteinR constructs for 30 min at RT, and subsequently added to mAmetrine-labeled *S. aureus* Newman- spa/sbi-KO. Bound anti-WTA was detected with goat-anti-human-kappa-Alexa$^{647}$. Samples were fixed with 1 % paraformaldehyde and analysed by flow cytometry using the mAmetrine fluorescence to gate on bacteria versus background signals.

### Anti-WTAIgG1/IgG3-Mediated neutrophil phagocytosis assay

Human neutrophils were isolated from heparinized blood using Ficoll-Histopaque gradient centrifugation as described (19). Anti-WTA IgG1/IgG3-mediated neutrophil phagocytosis assay was determined by flow cytometry as previously described (22). Briefly, different ProteinR constructs with fixed concentration were incubated with various concentrations of human anti-WTA IgG1/IgG3 for 15 min at RT while shaking. Next GFP-labeled *Staphylococcus aureus* Newman Spa/Sbi-KO were added and incubated for 15 min at 37°C while shaking. Subsequently, neutrophils were added resulting in a ratio of 10 bacteria per cell and incubated for an additional 15 min. The samples were fixed with 1 % PFA to stop the phagocytosis process. Flow cytometry was used to measure the neutrophil associated fluorescence. The data were analyzed with FlowJo_V10 and expressed as MFL of the neutrophil population relative to the IgG only control value.

### Neutrophil ROS production induced through immune complexes

Immunoglobulins were pre-incubated with 10 μg/ml of ProteinR constructs in a white 96-well plate for 15 min at RT. Isolated neutrophils were resuspended in IMDM + 0.1% HSA and 50 μl (1.25 x $10^6$ cell/ml) added to wells in the presence of 100 μl of pre-warmed HBSS containing 150 μM luminol and 0.1% HSA. The production of ROS was measured as chemiluminescence at 1 min intervals in a Centro LB 960 Microplate Luminometer. The level of ROS production was determined as Relative Light Unites (RLU) during 60 min.

### Binding to IgG coated Tosyl-beads

Magnetic Tosylactivated beads (Dynabeads) were coated overnight at 4°C while shaking with IgG, ± 1 µg per µl beads in 0.1 M Na-phosphate buffer plus 1 M ammonium sulphate. Beads were washed with PBS, blocked with 5 mg/ml HSA for 2 hours, and finally resuspended in PBS+0.1% HSA at ± 5 x $10^7$ beads/ml. In a round bottom microplate, 5 µl coated beads are mixed with fluorescent ligands and/or competitor proteins in a total volume of 30 µl on a shaking platform (900 rpm) at RT. Beads were washed with 200 µl buffer, resuspended in 150 µl and analyzed by flow cytometry. To verify IgG coating, beads were stained with a F(ab')2 goat-anti-human-Kappa-Alexa$^{647}$.

We used ProteinR-ΔC, D145 and D1, and Spa single domain B as wt (Spa-B-wt), only Fab binding variant (Spa-B-KK) and only Fc binding variant (Spa-B-AA) (23). All ligands were used at a concentration of 3 µg/ml for binding and 2 µg/ml in competition experiments. Non-fluorescent competitor proteins were pre-incubated at 20 µg/ml with coated beads for 30 min, followed by an additional 30 min incubation with FITC-labeled protein without wash step. After a final wash, beads associated fluorescence was measured by flow cytometry by gating on the single bead population.

## Results

### Identification , design, expression and purification of protein

Regulation of the immune system in a complex environment as the gut microbiome is extremely complex and not well understood. We therefore set out to identify bacterial proteins from the microbiome that interact with the immune system. We developed a phage display library of the human intestinal microbiome by using feces from 9 healthy volunteers to extract bacterial DNA. Several lead candidates were identified via selections against various immunologically relevant targets and using both low and high-throughput sequencing analysis as described in our previous studies (17, 19). More specifically, we randomly selected 48 colonies after three rounds of selection on C3 for sequencing. Data showed that there were 30 genes out of 48 colonies encoded by 20 different microorganism, indicating the high complexity and diversity of our phage library (Table 1). A BLAST search for 5 unique overlapping sequences show that they were encoded by multiple gut microorganisms with up to 80% homology. Most belong to different *Ruminococcus* species and one sequence annotates to *Blautia sp. Marseille-P2398*. The overlapping sequences showed a high homology to a secreted hypothetical protein RSAG_03154 from *Ruminococcus sp.* 5_1_36BFAA, but also to two individual secreted hypothetical proteins WP_062807200.1 and WP_062807201.1 from *Blautia sp. Marseille-P2398*.





**Table 1. Hits of phage display selection on C3**

| Sample | Protein name | Bacterial species |
|---|---|---|
| A1 | Peptidase M23B 56aa | Oceaniovalibus guishaninsula (proteobacteria) |
| A2 | Hypothetical protein (RSAG_03154) | Ruminococcus sp. 5_1_39BFAA (firmicutes) |
| A3 | Hypothetical protein | Roseburia sp. CAG:100 (fimicutes) |
| A4 | Cytosine-specific methyltransferase | Ruminococcus sp. CAG:579 |
| A6 | Aanaerobic ribonucleoside-triphosphate reductase | Eubacterium rectale DSM 17629 |
| A7 | Hypothetical protein (RSAG_03154) | Ruminococcus sp. 5_1_39BFAA (firmicutes) |
| A9 | Hypothetical protein (RSAG_03154) | Ruminococcus sp. 5_1_39BFAA (firmicutes) |
| A10 | Iditol 2-dehydrogenase | Rhizobium gallicum |
| B3 | Hypothetical protein (RSAG_03154) | Ruminococcus sp. 5_1_39BFAA (firmicutes) |
| B4 | Hypothetical protein | Prevotella sp. CAG:386 (Bacteroidetes) |
| B5 | vTC domain protein | Clostridium sp. CAG:448 |
| B6 | Glutamate N-acetyltransferase | Xenococcus sp. PCC 7305 |
| B7 | Leucyl-tRNA synthetase | Firmicutes bacterium CAG:103 |
| B8 | Glycoside hydrolase family 43 | Firmicutes bacterium CAG:272 |
| B9 | Leucyl-tRNA synthetase | Clostridium sp. CAG:138 |
| B10 | Cell divisionFtsK/SpoIIIE | Akkermansia muciniphila ATCC BAA-835 |
| B12 | Hypothetical protein | Bifidobacterium stellenboschense |
| C1 | Exinuclease ABC subunit | Enterorhabdus mucosicola |
| C2 | Hypothetical protein | Prevotella sp. CAG:873 |
| C4 | Hypothetical protein | Prevotella sp. CAG:873 |
| C6 | Hypothetical protein (RSAG_03154) | Ruminococcus sp. 5_1_39BFAA (firmicutes) |
| C7 | Hypothetical protein | Faecalibacterium sp. CAG:74 |
| C9 | Hypothetical protein PAV_16c00470 | Paenibacillus alvei DSM 29 |
| C12 | 1-deoxy-D-xylulose-5-phosphate synthase | Alistipes senegalensis |
| D2 | Hypothetical protein | Firmicutes bacterium CAG:65 |
| D3 | ATP-binding protein (109 aa) | Clostridiales |
| D4 | ATP-binding protein (109 aa) | Clostridiales |
| D9 | Ooxidoreductase | Rhizobium leguminosarum |
| D10 | Hypothetical protein | Eubacterium sp. CAG:86 |
| D11 | Zinc ABC transporter substrate-binding protein | Bacillus |

Sequence alignment revealed that WP_062807200.1 and WP_062807201.1 both overlap with RSAG_03154. Therefore, it is more likely that WP_062807200.1 and WP_062807201.1 may actually act as one protein like RSAG_03154. Nevertheless, we identified a conserved protein from multiple gut microorganisms.

We renamed this conserved protein as ProteinR. ProteinR has a molecular weight of approximately 62.7 kDa and is composed of a short N- terminal region, five repeat domains, and a relatively long C-terminal tail. Sequence alignment of the single domains D1, D2, D3, D4, and D5 reveals that D1 and D2 are identical, while the remaining three domains show a high similarity to the first two repeat domains except for a few amino acids (Fig S1). To better characterize ProteinR function, recombinant proteins including all the variants and single domains were made based on the sequence of RSAG_03154, containing a C-terminal His tag were purified from

*E.coli*. The 3D structure of ProteinR was predicted by I-TASSER (24–26). For additional cloning information on all the constructs used in this study see Fig S1.

## ProteinR binds to different immunoglobulin isotypes from different species

We verified that recombinant ProteinR indeed binds to human C3 using an ELISA (Fig. S2). Further experiments focused on revealing the function and mechanism of action of this protein. Surprisingly, in addition to binding to C3, ProteinR showed the ability to bind to human IgA, IgG, and IgM (Fig. 1A) as well. In order to determine which part of ProteinR contributes to Ig binding, single domains D1, D3, D4, and D5 were purified. There appeared to be no difference between the single domains and ProteinR in binding affinity to human secretory IgA and IgM. In contrast, multiple domains seems to be required for optimal serum IgG binding.

In addition to the binding of ProteinR to human Igs, we tested binding to Ig's from other species. ProteinR bind to mouse serum derived IgG and IgM and rabbit IgG in a similar binding pattern as to human IgG and IgM respectively (Fig 1B), ProteinR however was not able to bind to goat and sheep IgG (Fig 1B). Of note, the lack of binding to goat IgG is important in our experiments since most ELISA HRP-conjugates were of goat origin.

## ProteinR binds to different human immunoglobulin subtypes

To test whether ProteinR is able to bind different subtypes of human immunoglobulins and which domain(s) of ProteinR is/are responsible for this binding, we used human recombinant monoclonal immunoglobulins of different subtypes. ProteinR showed binding to all tested human anti-WTA (clone #4497) monoclonal immunoglobulins, IgG1, IgG2, IgG3, and IgG4 subclass as well as IgM and IgA1(Fig 2A). Remarkably, testing several other human monoclonal IgGs revealed that ProteinR does not bind to all the monoclonal IgGs tested. For instance, no binding was observed with any of the four IgG subclasses of an anti-StrepTagII or anti-DNP human mAb (Fig 2A and Table S1). Overall , Fig 2A reveals that all constructs of ProteinR bind to different anti-WTA monoclonal Igs showing the same binding pattern as we observed for human serum Igs. ProteinR showed no binding affinity difference between all the different IgG subtypes. However, we do observed that there is significant binding affinity difference to different subtypes of IgG among the individual ProteinR domains. D1 showed less affinity to all the IgG subtypes compared to the other individual domains D3, D4 and D5 which showed similar binding ability to each IgG subtypes. We supposed that a certain number of domains is needed for efficient IgG binding. To test this hypothesis, we compared constructs with multiple domains (D12, D123 and D1234, all without N-terminal domain) with single domain (D1 and D3, all with

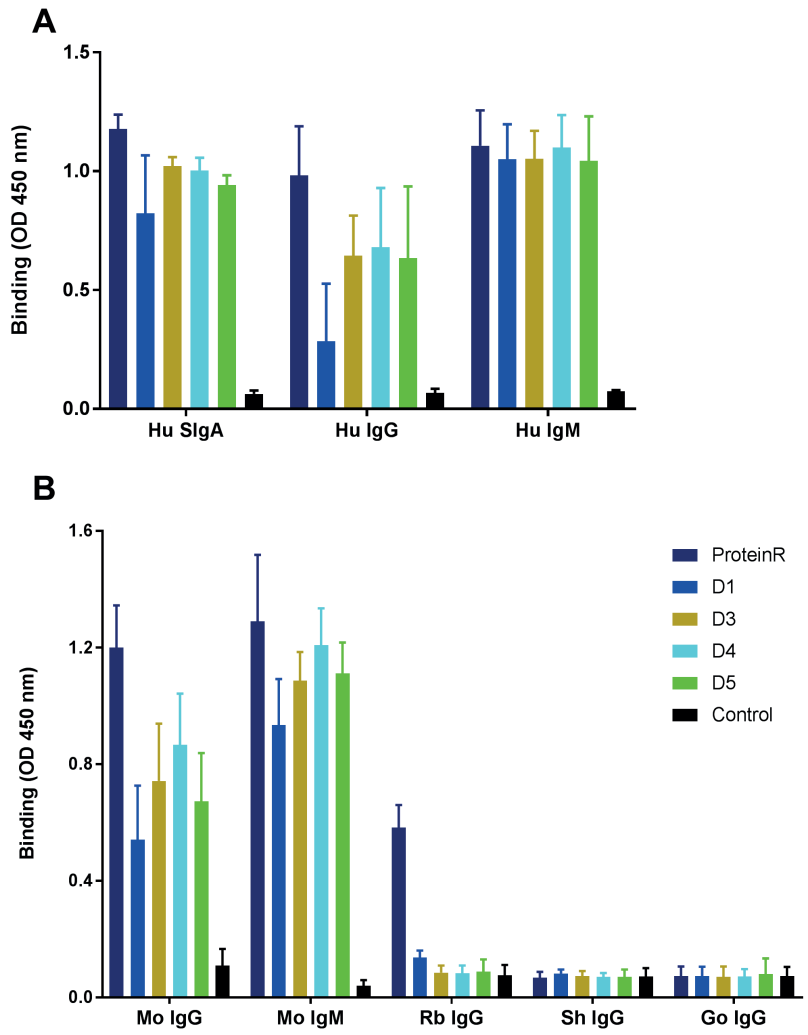
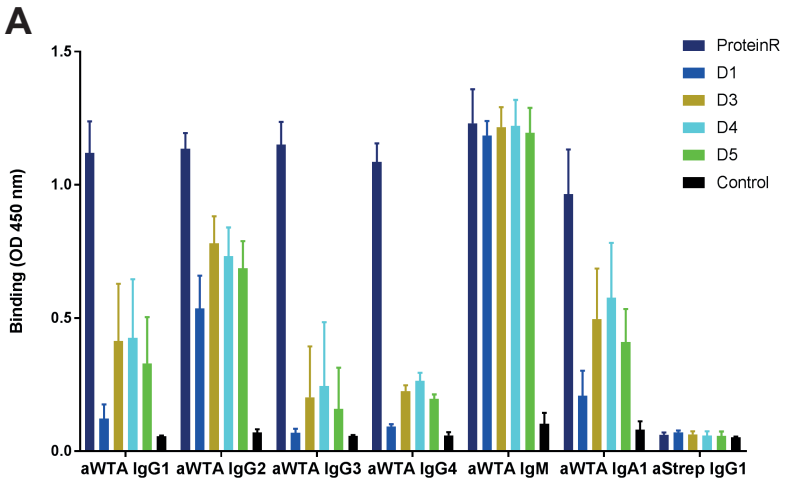



**Figure 1. ProteinR binds to different immunoglobulin isotypes from different species.** (A) ProteinR constructs (3 µg/ml) were coated in a 96-well plate and the binding of 1 µg/ml human Igs was detected using specific HRP-labeled goat anti-Igs. (B) The same procedure was applied for binding of mouse Igs and IgG of rabbit, goat and sheep. Data represents mean +/- SEM of three independent experiments.

N terminal domain) on IgG subtypes. The results showed that at least three domains are needed for efficient IgG binding while the N-terminal domain is not required for IgG binding (Fig 2B-2E). We also observed that all the constructs showed better binding affinity to IgG2, especially, for the single domain D1. To further evaluate the contribution of the different domains, additional constructs were generated



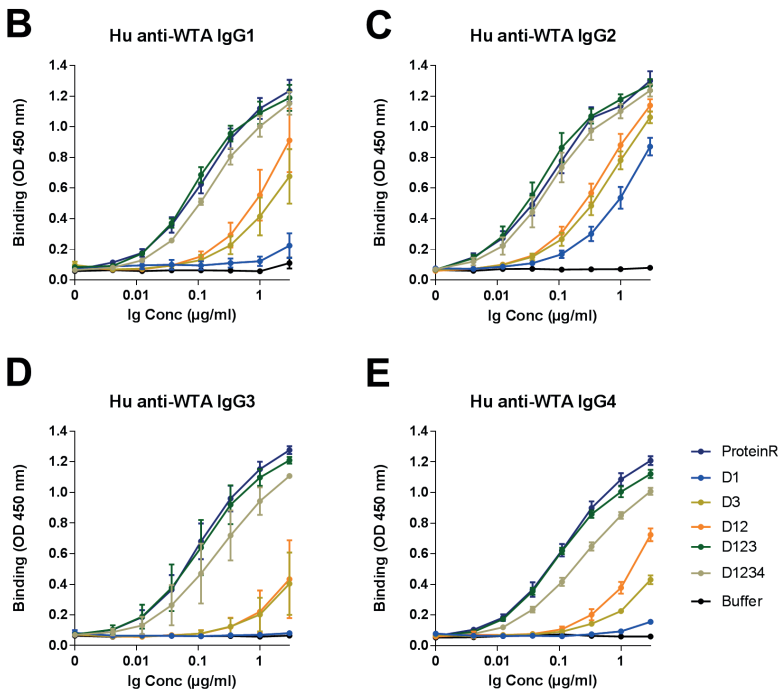




**Figure 2. ProteinR binds to different human immunoglobulin subtypes.** (A) ProteinR constructs (3 µg/ml) were coated in a 96-well plate and binding of different anti-WTA (clone #4497) and anti-StrepTagII monoclonal human Igs at 1 µg/ml was detected via HRP-labeled goat anti human Igs. (B-E) Binding of full concentration range of human anti-WTA (B) IgG1, (C) IgG2, (D) IgG3, and (E) IgG4 (clone #4497) to ProteinR constructs. IgG binding was detected via HRP-labeled goat anti human IgG. Data represents mean +/- SEM of three independent experiments.





with different combinations of 3 or more domains: D345, D145, and ProteinR-ΔC (truncated ProteinR without the C-terminal tail, which is equivalent to D12345 plus N-terminal domain). D345, D145, and ProteinR-ΔC all showed comparable binding with the three domains construct D123 and the four domains construct D1234. All multiple domain constructs showed better binding than one single domain or two domains, which confirmed that any combination of three or more domains is sufficient for efficient IgG binding, while the C-terminal and N-terminal domains are not required for binding (Fig S3). Collectively, ProteinR binds to different human Ig classes , whereby any single domain is sufficient for IgM and IgA binding while the IgG subclasses interaction depends on the domain while any of 3 domains are required for efficient binding. The N-terminal and C-terminal domain of ProteinR are not required for Ig binding.



## ProteinR binds to F(ab')2 region of IgG and interferes with epitope recognition

Interestingly, the complete lack of binding between ProteinR and anti-StrepTagII IgGs as opposed to anti-WTA IgGs (clone #4497) that share the same IgG subclass backbone, indicate that ProteinR may interact with the Fab region of immunoglobulins. Therefore, Fc and F(ab')2 fragments from both human serum IgG and monoclonal anti-WTA IgG2 were tested for ProteinR binding. Fig 3A showed that ProteinR binds to F(ab')2 of anti-WTA IgG2 (clone #4497) and human serum IgG, but not to their Fc fragments. D345, ProteinR-ΔC and ProteinR-ΔN show the same results as full length ProteinR. Beside this, purified F(ab')2 of the anti-StrepTagII IgG2 was not recoginized by ProteinR in accordance with the lack of binding to the intact IgG2. Unexpectedly, D1 showed no binding to F(ab')2 of anti-WTA IgG2, even though D1 showed binding to intact anti-WTA IgG2. Moreover, recombinant Fab fragment of anti-WTA IgG was produced and tested in this binding assay. Surprisingly, only ProteinR and ProteinR-ΔN are able to bind recombinant Fab fragment of anti-WTA IgG (Fig S4). Since all recombinant human IgG constructs share the same backbone of the Kappa light chain and constant regions of the heavy chain (for each IgG subclass), we examined the $V_H$ composition of our ProteinR-binding and -nonbinding recombinant human monoclonal IgGs using the website of the International Immunogenetics Information System (http://www.imgt.org/). Our data indicated that ProteinR binds to the antibodies independently to $V_H$ subgroup (Table S1). To summarize, ProteinR binds to Fab region of Igs, but not Fc.

A well-known Ig binding protein with homologous repeating domains is protein A (SpA) produced by *Staphylococcus aureus*. Spa is a 42-kDa protein that contains five highly homologous extracellular Ig-binding domains E, D, A, B, and C, that bind to both Fc and Fab part of Igs (27, 28). The interaction of Spa with the IgG

Fab is restricted to the variable region of the Fab heavy chain 3 ($V_H3$), without the involvement of the hypervariable regions implicated in antigen recognition (29, 30). To investigate whether an immunoglobulin bound to ProteinR still can bind its epitope, binding and competition were performed on mAmetrine-labeled *S. aureus* Newman- spa/sbi-KO. The first experiment was set up to determine binding of ProteinR to the bacterial surface bound anti-WTA IgG1 antibody using a fluorescent labeled anti-His-Tag antibody. Spa-B (B-domain of Spa, also with an C-terminal His-Tag) as positive control, ProteinR, ProteinR-ΔN, ProteinR-ΔC, and D345 all bind dose dependently to the anti-WTA decorated bacteria while the single domain D1 did not (Fig 3B). To evaluate potential inference of ProteinR with antibody epitope recognition, the same anti-WTA IgG1 was preincubated with ProteinR or variants before binding to the bacterial surface. Bound anti-WTA IgG1 (clone #4497) was detected with a fluorescent labeled Goat-anti-Human-Kappa chain. All ProteinR constructs interfered with anti-WTA IgG binding to *S. aureus*, while D345 was less effective; Spa-B did not interfere with IgG binding (Fig 3C). The same set of experiments, binding of ProteinR to bound IgG and prevention of IgG binding by preincubation, were repeated with anti-WTA IgG3 (clone #4497) and showed the same results (Fig S5A and S5B). As an additional control we used another anti-WTA IgG1 (clone #6297) which is a ProteinR non-binder (Table S1), and showed indeed no interference with anti-WTA IgG binding to the bacterial surface (Fig S5D). Taking all together, ProteinR binds to Ig via the F(ab')2 fragment and interferes with antigen recognition.

## ProteinR inhibits Fc-mediated phagocytosis

Neutrophils, the most abundant and efficient phagocyte, play a key role in the host defense bacterial infections (31, 32). Optimal neutrophil phagocytosis requires opsonization, a process in which the bacterial surface is decorated with molecules, called opsonins, that are recognized by specific neutrophil surface receptors. Immunoglobulins is one of the most abundant and efficient opsonins in serum. They recognize antigens via Fab's variable region and bound antibodies are detected by neutrophils via interaction with Fc Receptors (FcRs) via its Fc region. In particularly, FcγRIIa and FcγRIIIb recognize IgG mediated opsonization, FcαRI recognizes IgA mediated opsonization. Spa inhibits phagocytosis of *S.aureus* by binding to the Fc region of antibodies (33). Because ProteinR is able to interfere with the antigen recognition site of IgG, phagocytosis assays were performed with human neutrophils and GFP-labeled *S. aureus* Newman-spa/sbi KO to examine if ProteinR can inhibit Fc-mediated phagocytosis. Human anti-WTA IgG1 clone #4497 was used to initiate phagocytosis of *S. aureus* by neutrophils and different constructs of ProteinR were added to evaluate potential interference. Conform







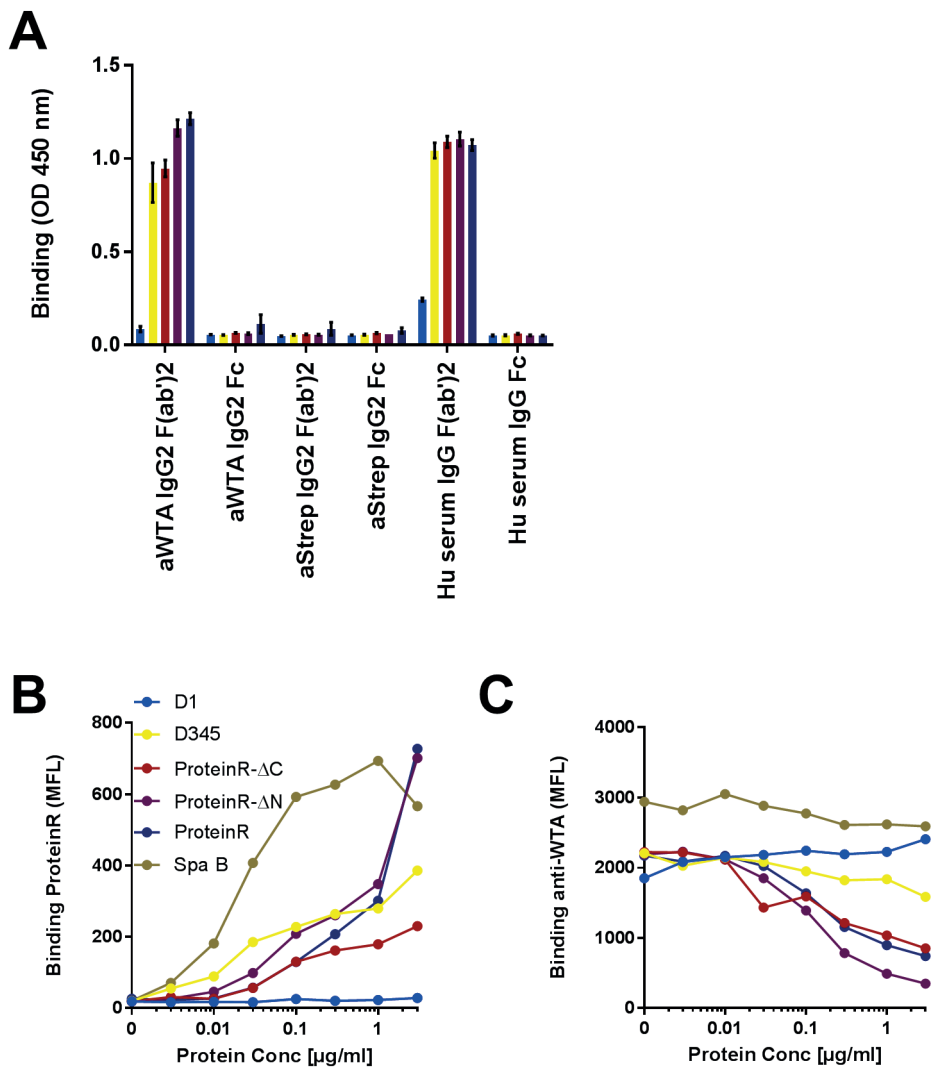



**Figure 3. ProteinR binds to F(ab')2 region of IgG and interferes with epitope recognition.** (A) ProteinR constructs (3 µg/ml) were coated to a 96-well plate and subsequently incubated with 1µg/ml different immunoglobulin fragments. Binding was detected with the appropriate secondary HRP conjugated immunoglobulins. Data represents mean +/- SEM of three independent experiments. (B) Binding of different ProteinR constructs to S. aureus bacteria coated with 2 µg/ml anti-WTA IgG1 (clone #4497) was determined with goat-anti 6x His tag followed by PE conjugated Donkey anti-goat IgG. (C) Anti-WTA IgG1 (clone #4497) was preincubated with different concentration of ProteinR or variants before binding to the bacterial surface. Bound anti-WTA IgG1 was detected with Alexa647 labeled Goat-anti-Human-Kappa chain. Data represents mean fluoresces of three independent experiments.

literature, Spa-B effectively inhibited the anti-WTA IgG1 mediated phagocytosis dose dependently (Fig 4A). Comparable with Spa-B, also ProteinR and ProteinR-ΔN potently inhibited phagocytosis, while three (D123), four (D1234), and fivedomains (ProteinR-ΔC) showed some inhibition at higher concentrations, and single domain D1 was ineffective (Fig 4A). This data match with the interference with anti-WTA IgG binding as shown in Fig 3 but also indicates that the C-terminal tail of ProteinR plays an important function during this process, while, the small N-terminus is not essential. Therefore, we constructed new ProteinR variants, including only the C-terminal region, one domain plus C-terminus (D5C) and three domains plus C-terminus (D345C), to figure out which part of ProteinR is essential for the functional activity. Unfortunately, we were not able to produce soluble C-terminus only. However, by comparing D5 with D5C and D345 with D345C, our data confirmed that only the presence of all domains (five) with a C-terminus is able to inhibit phagocytosis (Fig 5B). An additional phagocytosis experiment was conducted with human anti-WTA IgG3 clone #4497 and anti-WTA IgG1 clone #6297 (ProteinR-non binding IgG). Phagocytosis of *S. aureus* opsonized with a concentration range of anti-WTA IgG3 (clone #4497) showed the same pattern of inhibition by ProteinR constructs (at a fixed concentration of 10 µg/ml) as for anti-WTA IgG1 (fig S5C). Of note, since Spa-B does not bind human IgG3 Fc-region, no inhibition is observed. Phagocytosis mediated by the ProteinR non-binder anti-WTA IgG1 (clone #6297) was only inhibited by Spa-B, and not by ProteinR as expected (Fig S5E). Collectively, ProteinR inhibits Fc-mediated phagocytosis.

## The ProteinR immunoglobulin complex triggers neutrophil ROS production via Fc-receptors

Triggering of Fc-receptors on neutrophils, both FcγRIIA, FcγRIIIB, and FcαRI, also induces ROS production that can be induced by immune complexes(34–36). We therefore used luminol-dependent chemiluminescence to study potential ProteinR-antibody complexes formed by ProteinR and IgG or IgA. As expected, neutrophils treated with ProteinR plus MEDI4893 IgG1 or aWTA IgA1- led to a robust ROS production (Fig 6). The IgG-mediated activation can be inhibited with FLIPr-Like, an FcγR-blocking protein secreted by *S. aureus* (22). The IgA-mediated activation can be inhibited with SSL7, also a *S. aureus* secreted protein that blocks FcαRI (22, 37, 38). Omission of either ProteinR or IgG/IgA or exchange with ProteinR-nonbinding recombinant human monoclonal IgGs did not induce ROS production (data not shown), suggesting that binding of ProteinR to IgG or IgA formed ProteinR-antibody complexes, thereby have a powerful ability to modulate Fc-receptor mediated functions on neutrophils.





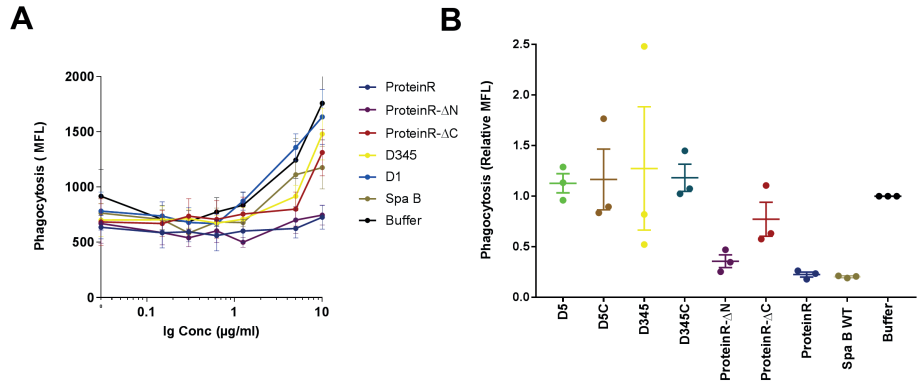



**Figure 4. ProteinR inhibits Fc-mediated phagocytosis.** Different ProteinR constructs (10 µg/ml) were incubated with (A) various and (B) fixed concentration (10 µg/ml) of human anti-WTA IgG1 (clone #4497). Next GFP-labeled S. aureus Newman Spa/Sbi-KO were opsonized, subsequently neutrophils phagocytosis was measured by flow cytometry. Data are expressed as MFL of the neutrophil population relative to the IgG only control value. Error bar are SEM of three biological replicates.

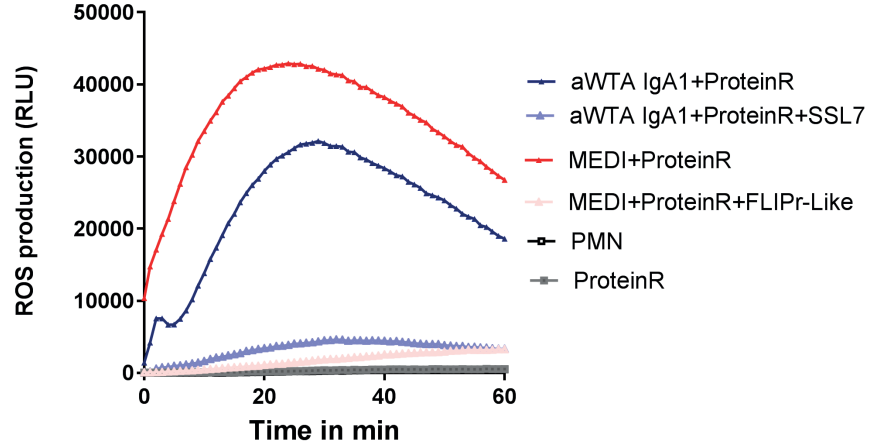



**Figure 5. The ProteinR immunoglobulin complex triggers neutrophil ROS production via Fc-receptors.** 10Immunoglobulins were pre-incubated with 10 µg/ml of ProteinR constructs in a white 96-well plate. Neutrophils were added and the production of ROS was measured as chemiluminescence at 1 minute intervals. The level of ROS production was determined as Relative Light Unites (RLU) during 60 min. Data from a representative experiment (1out of 3).

### ProteinR is able to compete with Spa-B

Results demonstrated that ProteinR acts as an Ig binder by interacting with the F(ab')2 fragment. Since Spa also binds to a defined region of the Ig Fab region, we performed selective competition between single Spa-B domains, ProteinR domain D145 and Protein L as a control Ig binder that binds specifically the Ig kappa chain. Therefore IgG was randomly coated onto tosyl-activated beads and used for competition for binding of the FITC-labeled ligands Spa-B-AA mutant (only binds to IgG Fc part), Spa-B-KK mutant (only binds to IgG Fab part), and D145. Initial experiments confirmed that IgGs were coated onto the beads and that FITC-labeled D145 and Spa-B-AA bind to both MEDI (clone #4893) IgG1 and anti-WTA IgG1 and IgG2 coated beads. FITC-labeled Spa-B-KK only bound to MEDI (clone #4893) coated

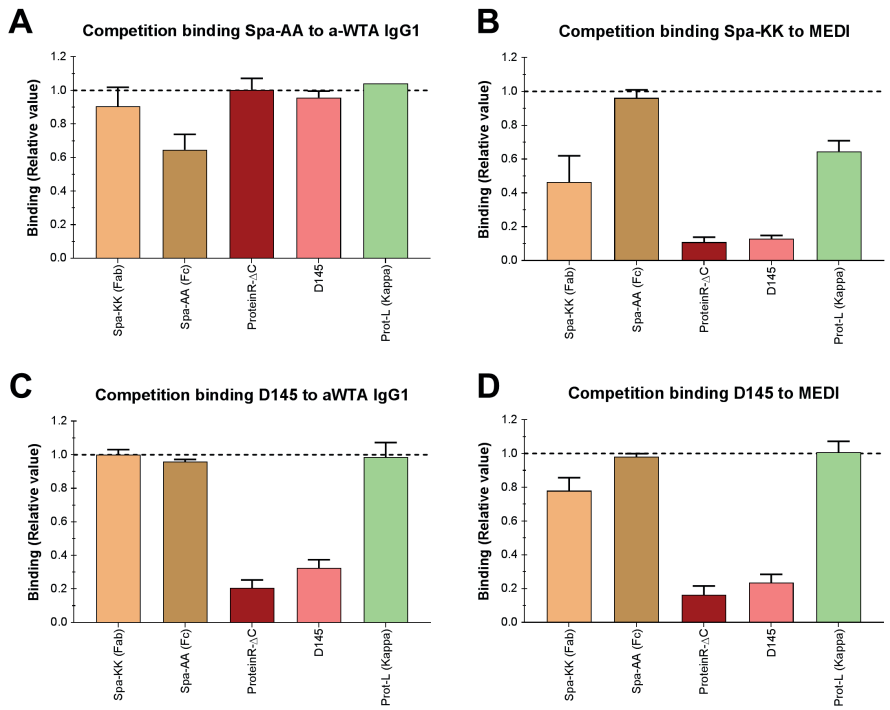



**Figure 6. ProteinR is able to compete with Spa-B.** (A) 20 µg/ml Spa-B-KK, Spa-B-AA, ProteinR-ΔC, D145 and Protein L were tested to compete with 3µg/ml of Spa-B-AA binding to anti-WTA IgG1 (clone #4497). (B) 20 µg/ml Spa-B-KK, Spa-B-AA, ProteinR-ΔC, D145 and Protein L were tested to compete with 3µg/ml of Spa-B-KK binding to MEDI (clone #4893). (C) 20 µg/ml Spa-B-KK, Spa-B-AA, ProteinR-ΔC, D145 and Protein L were tested to compete with 3µg/ml of D145 binding to anti-WTA IgG1 (clone #4497). (D) 2µg/ml Spa-B-KK, Spa-B-AA, ProteinR-ΔC, D145 and Protein L were tested to compete with 3µg/ml of D145 binding to MEDI (clone #4893). Fluorescence values are expressed relative to the ligand only binding with subtraction of background signal. Error bar are SEM of three biological replicates.





beads, confirming the specific heavy chain $V_H3$ annotation (Suvratoxumab with IGHV3-13*01, 96.9%; https://chem.nlm.nih.gov/chemidplus/unii/4L1997J4DF). The data demonstrated that unlabeled ProteinR-ΔC and D145 are not able to compete with FITC-labeled Spa-B-AA (specific for the Fc recoginition) to anti-WTA IgG1 (clone #4497) (Fig 6A), but ProteinR-ΔC and D145 are able to compete with FITC-labeled Spa-B-KK to MEDI (clone #4893) (Fig 6B). These data match with our data that ProteinR-ΔC and D145 bind to IgG is restricted to the F(ab')2 part, and does not involve the Fc part. In contrast, both Spa-B-AA and Spa-B-KK, as a negative/positive interference ligand, are unable to compete with FITC-labeled D145 to anti-WTA IgG1 (clone #4497) (Fig 6C) or to MEDI (clone #4893) (Fig 6D). As expected, ProteinR-ΔC is able to compete with FITC-labeled D145 binding (Fig 6C and 6D). As control we included Protein L (four domain protein) that specifically binds to Kappa light chains and hence showed no interference with any of the FITC-labeled ligands (39). In summary, constructs of ProteinR are able to compete with the single domain of Spa, but not vice versa.



## Discussion

In this study, a phage display library of the human intestinal microbiome was developed and used to identify potential proteins that interact with different immune components. In a selection on human C3, a multi-domain secreted protein from several gut microorganisms was identified and named ProteinR. Besides its interaction with C3, we characterized ProteinR as a novel immunoglobulin binder protein. The identification of an Ig binding protein in a phage selection on C3 is most likely caused by contaminating traces of Ig in the C3 preparation, because C3 was isolated from serum, which contain a large amount of immunoglobulins. Therefore, except for screening proteins that binds to C3, we may also fished out proteins that can interact with immunoglobulins. The problem of Ig-binders has been acknowledged by *C. Fevre et al* (17), where Spa was used to block the proteins purified from serum prior to *S.aureus* screening to prevent selection of phages displaying Spa, a well-known immunoglobulin binding protein secreted by *S. aureus*.

ProteinR binds to Igs from different species and also binds to different subtypes of IgG and IgA. This protein consists of 5 domains with a few amino acid differences, whereby domain 1 and 2 are duplicates, in between a N- and C-terminal tail. At least three domains are needed for optimal IgG and IgA1 binding, while a single domain is sufficient for IgM and secretory IgA binding (Fig 1A). N- and C-terminal tail of ProteinR are not necessary for intact Igs binding. ProteinR is not the first bacterial immunoglobulin binding protein. *S.aureus* secretes four immunoglobulin-binding

proteins, which are Protein A (Spa) (40), Staphylococcal binder of immunoglobulin (Sbi) (41) and Superantigen-like proteins 7 and 10 (SSL7 and SSL10) (38, 42). All of these proteins play a crucial role in virulence of *S. aureus* infection. The Sbi IgG-binding domains show significant homology to the IgG-binding repeats of Spa (43). Protein G, from group G streptococci, shows a unique and specific affinity for the Fc region of mammalian polyclonal and monoclonal IgG. Protein A (Spa) and Protein G are structurally very similar, both of them have been extensively characterized and turned into a standard IgG purification tool (44). But they have slightly different affinities for IgG subclasses across different species. These affinities overlap, but in general, Spa has greater affinity for rabbit, pig, dog, and cat IgG whereas protein G has greater affinity for mouse and human IgG. The light chain-binding protein (protein L) from some strains of *Peptostreptococcus magnus*, specifically interacts with kappa light chains without affecting the antigen-binding site of antibodies, and has been developed as a immunoglobulin detection and purification tool (45). ProteinR showed some similarity with Spa, Protein G and Protein L, as it contains multiple homologues domains and binds to multiple Igs via F(ab')2. Strangely, one single domain of ProteinR such as D1 is not enough for binding to F(ab')2 part of IgG2, even though D1 shown binding ability to intact IgG2. Moreover, only the constructs of ProteinR which contain C- terminus (D5C, D345C, ProteinR-ΔN, ProteinR), are able to bind to single Fab (Fig S4). Taking all together (Table 2), we supposed that the natural conformation of two neighboring Fab fragments linked via the hinge region is required for proper ProteinR interaction without the need for its C-terminal part. The maximal binding for IgG requires 3 domains that probably effectively cover the distance in F(ab')2. The C-terminal region of ProteinR apparently compensates for this interaction with the Fab fragment. Furthermore our data shown that C-terminus is essential for Fc-activation as only ProteinR constructs that contain a C-terminus are able to induce ROS production (Fig S6). Unfortunately we were unable to directly assess any Ig binding property of the C-terminal region due to failure in protein expression. Competition with Spa-B shown that ProteinR is able to compete Spa, but not verse vice. This could be due to the fact that Spa-B single domain is not enough to compete with multiple domains of ProteinR. However, this also gives us the clue that beside binding to VH region, ProteinR may also bind to the hinge region of IgG. In contrast to Spa, Protein G and Protein L, ProteinR lacks the LPXTG motif in its C-terminal domain and acts as a secreted protein instead of surface expressed. Remarkably, ProteinR interferes with the binding of an immunoglobulin to its epitope and inhibits neutrophil phagocytosis by competition with the antigen-binding site of the antibodies. Additionally it induces neutrophil ROS production by forming an ProteinR-antibody complexes with IgG/IgA, a property that has not been shown for Spa, Protein G and Protein L. It is interesting that Protein R on one hand interferes with phagocytosis and simultaneously mediates neutrophil activation and induces ROS production. The reason why protein mediates two opposite neutrophil effector function via binding immunoglobulins could because that the different Fc receptors are involved, FcγRIIa mainly mediates phagocytosis, while FcγRIIIb mainly mediates ROS production (46) .





**Table 2. ProteinR construct binding ability and function.**

| Protein Constructs | Intact IgG | via F(ab')2 | Singel Fab | Fc-mediated phagocytosis | Fcα-mediated ROS |
|---|---|---|---|---|---|
| One domain plus N terminus | + | - | - | - | - |
| One domain plus C terminus | +++ | +++ | ++ | - | +++ |
| Three domains | +++ | +++ | - | - | - |
| Three domains plus C terminus | +++ | +++ | ++ | - | +++ |
| ProteinR- ΔC( five domains) | +++ | +++ | - | ++ | - |
| ProteinR- ΔN( five domains plus C terminus) | +++ | +++ | ++ | +++ | +++ |
| ProteinR( full length) | +++ | +++ | ++ | +++ | +++ |

*Lactobacillus reuteri* mucus-binding protein (MUB) is a cell-surface protein that is involved in bacterial interaction with mucus and colonization of the digestive tract. MUB contains two different types of repeats (Mub1 and Mub2) present in six and eight copies, respectively, and shown to be responsible for the adherence to intestinal mucus. The closest structural homologue according to the I-Teaser to the domains of ProteinR is the B1-domain of MUB-R5. Mub-R5 B1 domain reveals an unexpected functional immunoglobulin binding activity, and the N-terminal domain shows structural similarity to the repeat unit of Protein L, suggesting binding in a non-immune Fab-dependent manner (47). The binding of individual repeats (Mub-R5, Mub-R6, and Mub-RI) showed similar binding patterns to Igs but with variable affinities. However, unlike ProteinR binding property, the triple repeat Mub-RI–III did not show any synergistic effect on binding. The presence of immunoglobulin binding protein on the surface of gut microbiota, including *E. coli*, has also been documented. The immunoglobulin-binding proteins from *E. coli* (Eibs) bind human IgG Fc (48). However, to elucidate what is the actual function of immunoglobulin binding in the gut, further studies need to be performed. All those immunoglobulin binding proteins present in the gut may have significant biological implications. Secretory Igs such as IgA, IgM, and IgG that are present in mucosal surfaces potentially provide a first line of defense against microorganisms

(49). Especially, secretory IgA play a key role in preventing pathogen adherence to the gastrointestinal surfaces (50). The attachment of bacteria to gastrointestinal surfaces may influence the host health by affecting the local microbial composition or by the stimulation of the gastrointestinal immune system. Compared to Mub and Eibs, ProteinR shows binding to Igs from more species and also interferes with antigen recognition via binding to the Fab region(s). In addition, the intriguing observation that ProteinR like genes were found in different commensal bacterial species suggests that this protein may play an pivotal role in gut colonization and keeping host-microbiome balance. All those evidence reveal that ProteinR may play a powerful role not only for antibody purification, but also may contribute to balance the colonization of commensal gut microbiome.

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# Supplement data

**Table S1. Information of used Monoclonal immunoglobulins during this research**

| Monoclonal immunoglobulins | Type | VH type | Target | Producer | OD (450 nm) |
|---|---|---|---|---|---|
| Hu α-WTA (#4461) | IgG1 | VH1 | Wall teichoic acid | UMCU | 1,215 |
| Hu α-WTA (#4624) | IgG1 | VH1 | Wall teichoic acid | UMCU | 0,186 |
| Hu α-StrepTagII | IgG1 | VH1 | StrepTag II- peptide | UMCU | 0,246 |
| Hu α-StrepTagII | IgG2 | VH1 | StrepTag II- peptide | UMCU | 0,305 |
| Hu α-StrepTagII | IgG3 | VH1 | StrepTag II- peptide | UMCU | 0,442 |
| Hu α-StrepTagII | IgG4 | VH1 | StrepTag II- peptide | UMCU | 0,082 |
| Hu α-PG (M130) | IgG1 | VH2 | Peptidoglycan | Genmab | 0,129 |
| Hu α-PG (M130) | IgG3 | VH2 | Peptidoglycan | UMCU | 0,071 |
| Hu α-WTA (#4497) | IgG1 | VH3 | Wall teichoic acid | UMCU | 1,34 |
| Hu α-WTA (#4497) | IgG2 | VH3 | Wall teichoic acid | UMCU | 1,308 |
| Hu α-WTA (#4497) | IgG3 | VH3 | Wall teichoic acid | UMCU | 1,338 |
| Hu α-WTA (#4497) | IgG4 | VH3 | Wall teichoic acid | UMCU | 1,043 |
| Hu α-WTA (#4497) | IgM | VH3 | Wall teichoic acid | UMCU | 1,712 |
| Hu α-WTA (#4497) | IgA1 | VH3 | Wall teichoic acid | UMCU | 1,155 |
| Hu α-WTA (#4497) | Fab | VH3 | Wall teichoic acid | UMCU | 0,592 |
| Hu α-WTA (#6292) | IgG1 | VH3 | Wall teichoic acid | UMCU | 1,269 |
| Hu α-WTA (#4462) | IgG1 | VH3 | Wall teichoic acid | UMCU | 1,244 |
| Hu α-GlcNac-SDR | IgG1 | VH3 | N- acetylglucosamine | UMCU | 0,063 |
| Hu α-Pneumo (Lambda) | IgG1 | VH3 | Pneumococcal capsular polysacharide 6B | UMCU | 0,549 |
| Hu α-LTA (A120) | IgG1 | VH3 | Lipoteichoic acid | Genmab | 1,206 |
| Hu α-PNAG (F598) | IgG1 | VH4 | Poly-N-acetylglucosamine | Genmab | 0,08 |
| Hu α-DNP | IgG1 | VH4 | Di-Nitro-Phenol | UMCU | 0,063 |
| Hu α-DNP | IgG2 | VH4 | Di-Nitro-Phenol | UMCU | 0,072 |
| Hu α-DNP | IgG3 | VH4 | Di-Nitro-Phenol | UMCU | 0,066 |
| Hu α-DNP | IgG4 | VH4 | Di-Nitro-Phenol | UMCU | 0,074 |
| Hu α-WTA (#6267) | IgG1 | VH5 | Wall teichoic acid | UMCU | 0,325 |
| Hu α-WTA (#6297) | IgG1 | | Wall teichoic acid | Genmab | 0,158 |
| Hu α-WTA (#6078) | IgG1 | | Wall teichoic acid | Genmab | 1,189 |
| Hu α-LPS (A6H5C5) | IgG1 | | Lipopolysaccharide | Genmab | 0,056 |
| Hu α-LPS (A6H5C5) | IgM | | Lipopolysaccharide | Genmab | 0,13 |
| Hu α-SCIN (6D4) | IgG1 | | Staphylococcal complement inhibitor | Groningen | 1,223 |
| Hu α-SRBC | IgA1 | | Sheep red blood cells | UMCU | 1,243 |
| Hu α-SRBC | IgA2 | | Sheep red blood cells | UMCU | 1,249 |

**Table S2. Information of used polyclonal immunoglobulins during this research**

| Polyclonal immunoglobulins | Producer | OD (450 nm) |
|---|---|---|
| Hu IgG (serum) | Jackson Immunoresearch | 1,353 |
| Hu IgG Fc fragment | Jackson Immunoresearch | 0,052 |
| Hu IgG F(ab')2 fragment | Jackson Immunoresearch | 1,032 |
| Hu IgG lambda light chain (serum) | Sigma | 1,401 |
| Hu IgM (serum) | UMCU serumpool | 1,671 |
| Hu IgA (serum) | Jackson Immunoresearch | 1,605 |
| Hu IgA (secretory) | Biorad | 1,728 |
| Mouse IgG (serum) | Jackson Immunoresearch | 1,171 |
| Mouse IgM (serum) | Southern Biotech | 1,699 |
| Mouse IgG2a | Southern Biotech | 0,068 |
| Mouse IgG2b | Southern Biotech | 0,615 |
| Mouse IgG3 | Southern Biotech | 0,231 |
| Mouse IgG1 | Southern Biotech | 1.201 |
| Rabbit IgG (serum) | Jackson Immunoreasearch | 1,356 |
| Sheep IgG (serum) | ICM | 0,07 |
| Bovine IgG (serum) | UMCU | 0,03 |

**Table S3. Information of used conjugates during this research**

| Conjugate | Target | Label | Producer |
|---|---|---|---|
| Goat a-Hu IgG | Human IgG | HRP | Southern Biotech |
| Goat a-Hu Fcγ | Human Fc (IgG) | HRP | Jackson Immunoresearch |
| Goat a-Hu IgM | Human IgM | HRP | Southern Biotech |
| Sheep a-Hu IgA | Human IgA | HRP | Nordic |
| Goat a-Hu kappa | Human kappa light chain | HRP | Southern Biotech |
| Goat a-Mo IgM | Mouse IgM | HRP | Southern Biotech |
| Goat a-Mo IgG | Mouse IgG | HRP | Biorad |
| Goat a- 6*His Tag | 6*His Tag | unlabeled | Abcam |
| Donkey a-Mo IgG | Mouse IgG | HRP | Jackson Immunoresearch |
| Donkey a-Ra IgG | Rabbit IgG | HRP | Jackson Immunoresearch |
| Donkey a-Sh IgG | Sheep IgG | HRP | Fluka Analythical |
| Donkey a-Go IgG | Goat IgG | HRP | Jackson Immunoresearch |

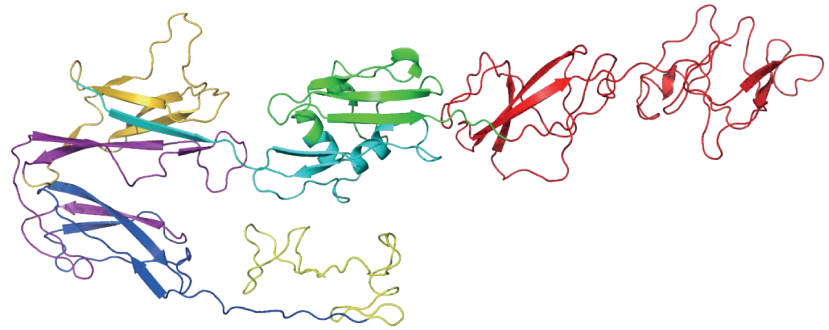




Below, the amino acid sequence of ProteinR with the differences aligned;

```
N-terminal MGLKERRKKIMALMLAACVAGTSVVPVAASDVSVFSDGTDASPEAFDSADVDSANGAA   58
D1   TKTVKINYYSEEEKKQIAEVEFTVDKDATSVNTSKLTAPEGYELVLSGDLAINDGYVYAAVRKVAT 124
D2   TKTVKINYYSEEEKKQIAEVEFTVDKDATSVNTSKLTAPEGYELVLSGDLAINDGYVYAAVRKVAT 190
D3   TKTVKINYYSEEEEKQIAEVEFTVDKDATSVNTSKLTAPEGYELVLSGDLAINDGYVYAAVRKVAT 256
D4   TKTVKINYYSEEEEKQIAEVELTVDKDATSVNTSKLTAPEGYELVLAGDLAINDGYVYAAVRKVAT 322
D5   TKTVKINYYSEEEEKQIAEVELTVDKDATSVNTSKLTAPEGYELVLSGDLAINDGYVYAAVRKAVT 388
     ************:*******:************************:****************..*
TKGITVVYQDRKGNVIKTAPMTVDKDATYINTGKLTAPDGYTIGIVGDIKISQNNKVYITVDQNKKSVTVI
FKESGKVVSSKKLSVVATAKYVNMSRITAPDRYQIVTTGSKLKISKNNTVTVEVKKLGKTIKVIYRLAKT
NKIVVTGEIVVDKKATSIKASEVPVPAGYKMVTTGSCSVKTKKIEIYVKK                    579
```


ProteinR structure predicted by I-TASSER



Note: Protein produced without signal peptide (MGLKERRKKIMALMLAACVAGT)

**Figure S1. ProteinR amino acid sequence , structure prediction and cloning information**

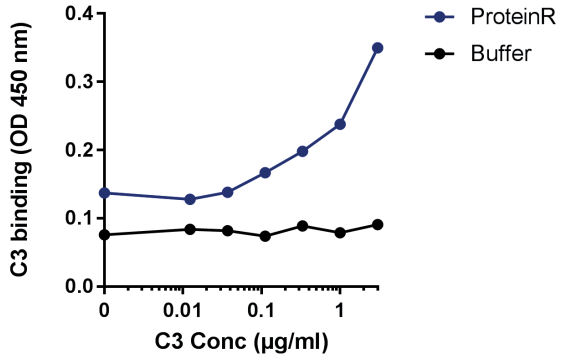



**Figure S2. ProteinR binds to C3.** ProteinR (3 µg/ml) were coated in a 96-well plate and the binding of various concentration of human C3 was detected using specific HRP-labeled goat anti-C3. Data from a representative experiment (1 out of 3).

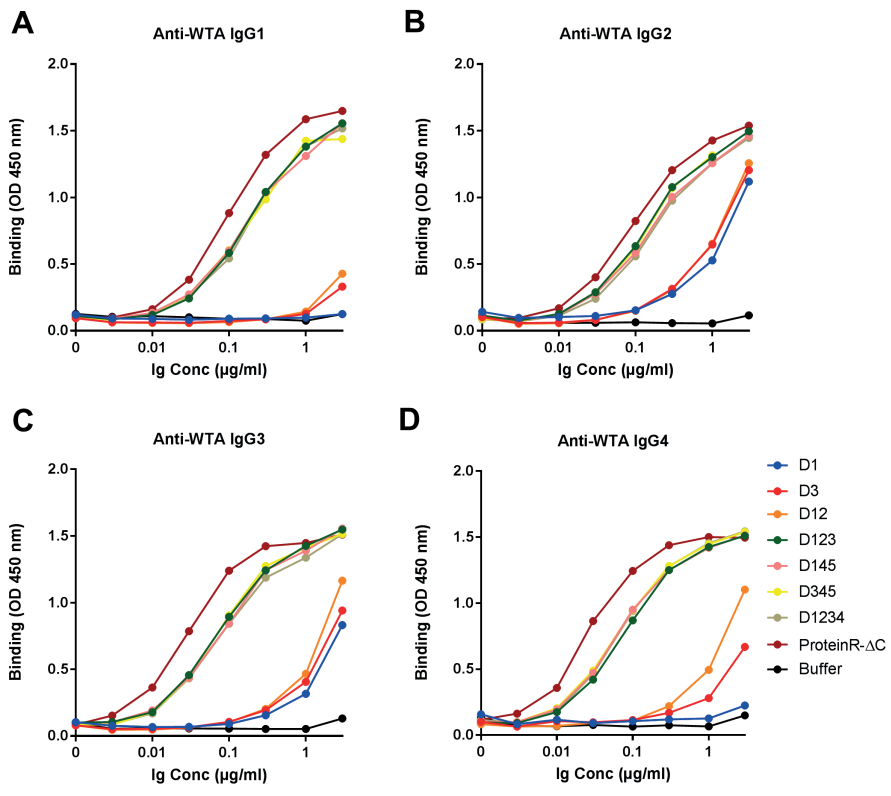



**Figure S3. Full concentration range of IgG binding to ProteinR.** 3 ug/ml of all the constructs of ProteinR were coated and incubated with a series concentration of human anti-WTA (clone #4497) (A) IgG1, (B) IgG2, (C) IgG3, (D) IgG4. Data from a representative experiment.





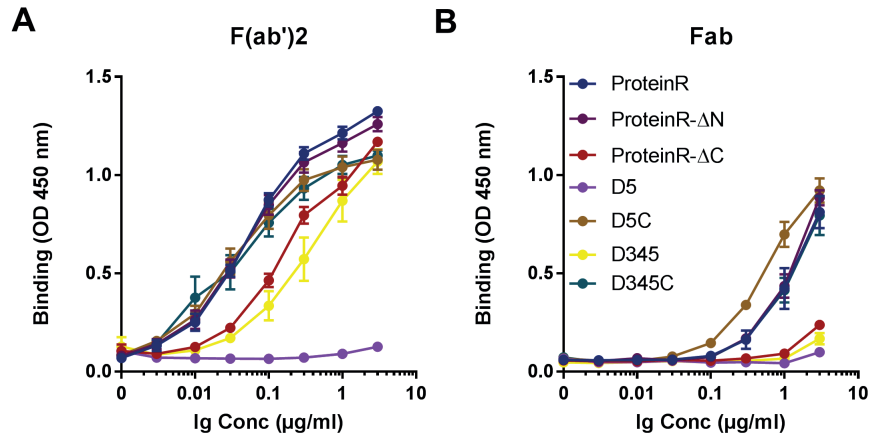



**Figure S4. C-terminus of ProteinR is important for binding single Fab.** ProteinR and variants which contain C terminus are able to bind both F(ab')2 from anti-WTA IgG2 and single Fab fragments from anti-WTA in a dose dependent manner. Error bar are SEM of three biological replicates.

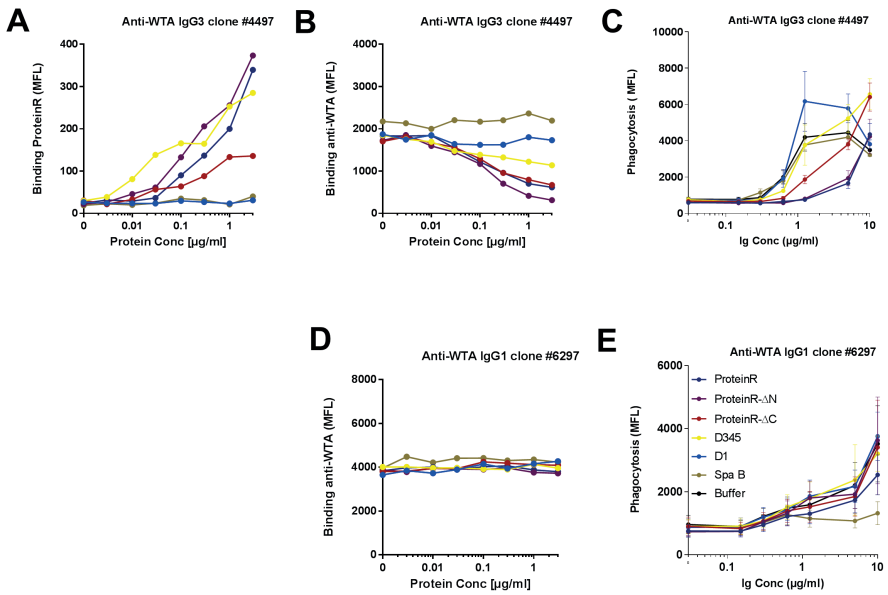



**Figure S5. ProteinR binds Fab and inhibits Fc-mediated phagocytosis.** (A) Binding of the different ProteinR constructs to bacterial surface bound anti-WTA IgG3 (#4497) were determined with goat-anti 6x His tag followed by PE conjugated Donkey anti-goat IgG. (B) Competition of the different ProteinR constructs with anti WTA IgG3 (#4497) binding to S.aureus were detected with goat-anti-human-kappa-Alexa647. Data represents mean of three independent experiments. (C) Different ProteinR constructs (10µg/ml) inhibit anti-WTA-IgG3 (#4497) mediated phagocytosis in a dose dependent manner. Data are expressed as MFL of the neutrophil population relative to the IgG only control value. Error bar are SEM of three biological replicates. (D) Competition of the different ProteinR constructs with anti-WTA IgG1 (#6297) binding to S.aureus were detected with goat-anti-human-kappa-Alexa647. Data represents mean of three independent experiments. (E) Different ProteinR constructs (10µg/ml) do not inhibit anti-WTA-IgG1 (#6297) mediated phagocytosis. Spa B inhibits anti-WTA-IgG1 (#6297) mediated phagocytosis in a dose dependent manner. Data are expressed as MFL of the neutrophil population relative to the IgG only control value. Error bar are SEM of three biological replicates.





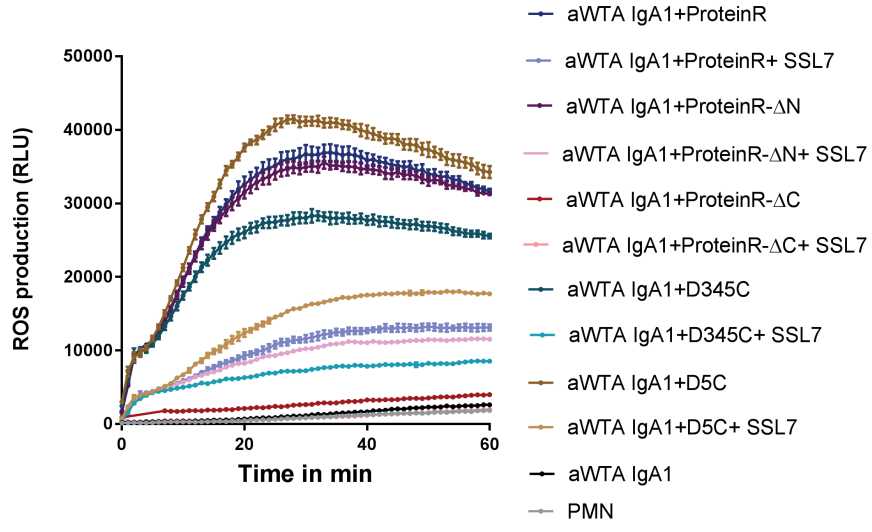



**Figure S6. ProteinR C terminal region is important for Fcα-mediated ROS production.** Anti-WTA IgA1 was pre-incubated with 10 µg/ml of ProteinR constructs in a 96-well white plate. The production of ROS was measured as chemiluminescence at 1 minute intervals. The level of ROS production was determined as Relative Light Unites (RLU) during 60 minutes. Data from a representative experiment.

CHAPTER 5

# Targeting the human C5a-Receptor: From virulence to therapy

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*Submitted for publication*

## Abstract

*Staphylococcus aureus* secretes an arsenal of virulence factors that target the human immune system. Some of these virulence factors interact with specific receptors present on human neutrophils. The secreted Chemotaxis Inhibitory Protein of Staphylococcus aureus (CHIPS), binds to the human complement component 5a receptor (C5aR), blocking the interaction with C5a and impeding neutrophil migration towards the site of infection. We hypothesized that CHIPS could be exploited to treat C5aR1 mediated diseases in humans. We show that administration of CHIPS in human C5aR1 knock-in mice successfully dampens C5a mediated neutrophil migration induced by a type 3 hypersensitivity reaction. We initiated a small phase-I trial to evaluate the potential use of CHIPS in human subjects. Even though CHIPS toxicology studies in animal models were successful, healthy human volunteers showed adverse effects directly after CHIPS administration. Subjects showed mild leukocytopenia and increased C-reactive protein concentrations, suggesting an inflammatory response. This is believed to be related to the presence of circulating anti-CHIPS antibodies in humans. The use of staphylococcal proteins, or other bacterial proteins, as therapeutics or immune-modulators in humans is possibly hampered by pre-existing circulating antibodies. Our data show the therapeutic potential of CHIPS as an anti-inflammatory agent in C5aR mediated diseases. Despite the unexpected safety issues in human subjects that temper the use of CHIPS in its current form as a therapeutic candidate, valuable pharmacokinetic and biological information was acquired from this pilot study.






## Introduction

The complement system is a major effector mechanism in the humoral immune defense (1, 2). The complement system is comprised out of more than 35 proteins that are present in host plasma and on cell surfaces. After activation of the complement system via either the classical, lectin or alternative pathway, a cascade of proteolytic events results in the release of multiple cleaved fragments of complement components (3, 4). The complement component C5a, a 74 amino-acid cleaved product of complement C5, is a powerful anaphylatoxin as well as a potent mediator of chemotaxis that interacts with the C5a-receptor 1(C5aR1). These receptors belong to the family of seven-transmembrane G-protein coupled receptors (GPCR), that are expressed on a wide variety of inflammatory cell (5–7). The interaction between C5a and C5aR1 results in the recruitment of phagocytes to the site of complement activation, linking the humoral immunity with the cellular innate responses (8).

It has become clear that C5aR1 is also involved in a variety of other inflammatory processes. Upregulation of C5aR1 on internal organs during the onset of sepsis, together with the excessive release of C5a, was proposed to lead to multi organ failure and death in rats (9, 10). Blockade of C5aR1 with polyclonal anti-C5aR1 antibodies was protective and increased survival in animal sepsis model (9). C5a and C5aR1 have also been described to be involved in disease processes such as ischemia-reperfusion injury, rheumatoid arthritis, asthma, immune complex diseases, neurodegeneration and Alzheimer's disease (11–14). Targeting of the C5aR1 has also shown to be beneficial in some of these disease processes in animals (15–19). Furthermore, inhibiting the proteolytic cleavage of C5 to C5a with Eculizumab, a monoclonal anti-C5 antibody, is used for the treatment of paroxysmal nocturnal haemoglobinuria, further emphasizing the relevance of the C5aR1 as a therapeutic target (20–22).

*Staphylococcus aureus* is a common commensal as well as a human pathogen that causes a variety of diseases, ranging from superficial skin and soft tissue infections to severe invasive infections with a poor prognoses and high mortality (23). Upon infection, *S. aureus* is faced with the host humoral and cellular innate immune responses resulting in the activation of phagocytes to contain the infection (4, 24). In return however, *S. aureus* has an arsenal of secreted virulence factors to evade the immune system (24, 25). One of these virulence factors is the Chemotaxis Inhibitory Protein of Staphylococcus aureus (CHIPS). CHIPS binds to the N-terminus of human C5aR1 with high affinity ($K_d$=1.1nM) and functionally blocks the interaction with C5a, thus preventing activation and antagonizing chemotaxis (26–28). These properties of CHIPS to inhibit the human C5aR1 with high specificity and affinity

makes it a promising candidate as an anti-inflammatory drug in diseases in which C5aR1 stimulation plays an important role. Previous studies have shown that the antagonistic activity of CHIPS on mouse C5aR1 is 30-fold lower compared to human C5aR1 expressing cells (26). This human specificity of CHIPS has hampered the assessment of CHIPS *in vivo* during inflammation and infection.

Here, we report the application of a transgenic human C5aR1 knock-in mouse (hC5aR1$^{KI}$) to assess CHIPS as a potential anti-inflammatory compound in C5aR1-mediated diseases. Furthermore, we investigate the safety and efficacy of CHIPS in a phase-I, randomized, double blind, placebo-controlled study.

## Results and discussion

### CHIPS binds hC5aR1$^{KI}$ murine neutrophils and inhibits stimulation by murine C5a

In order to validate the suitability of our hC5aR1$^{KI}$ mouse (29) as a model to evaluate CHIPS *in vivo*, we first assessed the activity of CHIPS on hC5aR1$^{KI}$ murine neutrophils. To this end, the binding of CHIPS on bone-marrow derived hC5aR1$^{KI}$ murine neutrophils was determined and compared to human neutrophils isolated from peripheral blood. We confirmed that CHIPS binds to hC5aR1$^{KI}$ murine neutrophils at comparable levels as human neutrophils (Figure 1a). To further assess the activity of CHIPS, inhibition of hC5aR1 was assessed on human and hC5aR1$^{KI}$ murine neutrophils. Wild-type (*wt*) murine neutrophils respond normally to mC5a but CHIPS is ineffective in inhibiting mC5a-mediated Ca-mobilization on these mC5aR expressing cells (Figure 1b). Correspondingly, CHIPS inhibition of mC5a mediated Ca-mobilization of bone-marrow derived hC5aR1$^{KI}$ neutrophils reflected that of human neutrophils isolated from peripheral blood (Figure 1b). Hereby, we confirm the binding and inhibition of hC5aR1$^{KI}$ murine neutrophils by CHIPS, proving that our hC5aR1$^{KI}$ mouse is a suitable model to assess CHIPS activity *in vivo*.

### CHIPS inhibits C5aR mediated neutrophil migration *in vivo*

To assess the *in vivo* therapeutic potency of CHIPS, the immune complex-mediated Arthus reaction model (30, 31) was used in hC5aR1$^{KI}$ mice. The resulting inflammatory response and neutrophil recruitment in the Arthus reaction is mainly C5a mediated. By simultaneously administering ovalbumin intravenous (i.v.) and rabbit anti-ovalbumin IgG intraperitoneal (i.p.), an immune complex mediated type 3 hypersensitivity reaction is induced that leads to the generation of C5a (30, 31). An Arthus reaction was successfully induced in hC5aR1$^{KI}$ mice as reflected by the influx of neutrophils to the peritoneal cavity (Figure 2a). Administration of CHIPS reduced the number of neutrophils recovered from the peritoneal cavity of hC5aR1$^{KI}$ mice (Figure 2a). Some mice that received CHIPS showed suboptimal inhibition of neutrophil migration, whereas a single mouse showed no evident decrease in neutrophils recovered compared to untreated mice (Figure 2a).

As *S. aureus* also colonizes rabbits (32), it is possible that the rabbit anti-ovalbumin IgG fraction used to induce formation of immune complexes also contains specific antibodies against CHIPS with potentially neutralizing capacities. To this end, we determined the presence of anti-CHIPS antibodies in the rabbit anti-ovalbumin IgG used. Although the rabbit IgG fraction did contain very low levels of anti-CHIPS antibodies (Figure 2b), the presence of these anti-CHIPS antibodies only slightly neutralized CHIPS *in vitro* and evidently did not neutralize CHIPS *in vivo* (Figure 2c, a). Taken together, our investigations demonstrate the therapeutic potential of CHIPS by inhibiting C5a-mediated neutrophil migration *in vivo* in hC5aR1$^{KI}$ mice after inducing an Arthus reaction.

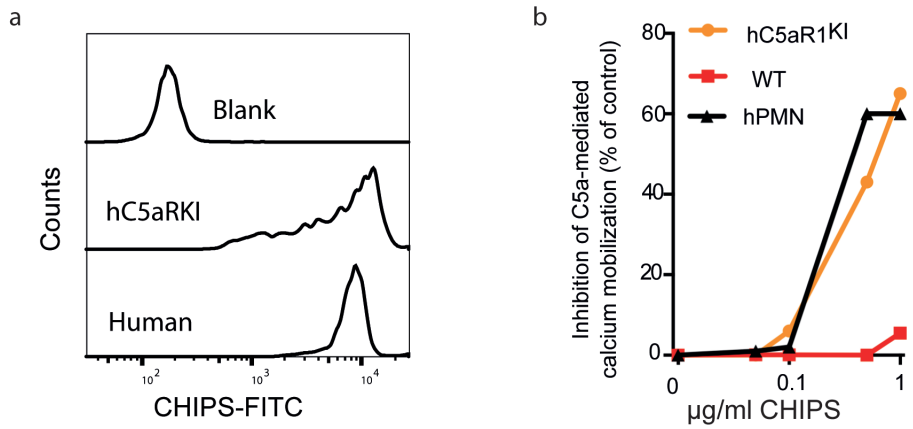


**Figure 1. CHIPS binds and inhibits hC5aR$^{KI}$ murine neutrophils comparable to human neutrophils.** Quantification of hC5aR1 expression in hC5aR1$^{KI}$ mice showed similar expression levels compared to human leukocytes (29). Furthermore, hC5aR1$^{KI}$ murine neutrophils responded normally to both murine C5a (mC5a) and human C5a as measured by calcium mobilization (29). (a) hC5aR$^{KI}$ bone marrow neutrophils and human blood neutrophils were isolated and incubated with 3µg/ml his-tagged CHIPS followed by anti-his-FITC antibodies. Cells were analysed by flow cytometry and the FITC fluorescent signal depicted as histograms. (b) As our hC5aR1$^{KI}$ murine model generates mC5a, the assessment of CHIPS inhibition was performed by mC5a stimulation. Bone marrow neutrophils of hC5aR$^{KI}$, wild-type mice and human neutrophils were pre-incubated with CHIPS at the indicated concentration and subsequently stimulated with murine C5a (10-8M). The basal fluorescence level was first measured for each sample before the addition of murine C5a. The C5a-mediated calcium influx was analysed by flow cytometry using FLuo-4AM. The average FLuo-4AM fluorescent signal was used to calculate CHIPS mediated inhibition of C5a responses. One experiment representative of two independent experiments is shown.

## CHIPS in human volunteers

*S. aureus* is commonly present as a commensal bacterium in humans and the *chp* gene is present in the majority of *S. aureus* strains. Consequently most, if not all humans, carry pre-existing anti-CHIPS antibodies (33–35). These anti-CHIPS antibodies present in human sera have been shown to interfere with CHIPS function *in vitro* (33). As a consequence, the presence of anti-CHIPS antibodies could neutralize CHIPS or induce an antibody-mediated immune reaction *in vivo,* hampering CHIPS function. To limit these undesired effects *in vivo*, only subjects with low anti-CHIPS titers were included in the study (antibody titer ≤ 3.92, as part of the exclusion criteria). To this end, we determined anti-CHIPS antibody titers in study subjects prior to receiving CHIPS. As expected, the 6 trail subjects have pre-existing anti-CHIPS antibodies (Figure 3a). To have an indication how subject titers relate to the general population, anti-CHIPS IgG titers were determined in sera collected from 168 human volunteers. As expected, anti-CHIPS IgG is detected in all 168 volunteers, resembling a Gaussian distribution (33) (Figure 3a). Accordingly, anti-CHIPS IgG titers from subjects were within the normal range of tested sera, representative of the anti-CHIPS IgG titers of the general population (Figure 3a). The anti-CHIPS antibody titers in subjects were considered low enough to not affect the safety assessment of CHIPS.

To further assess the safety of CHIPS, pre-clinical safety experiments were conducted in non-human subjects, prior to administration in humans. In all the animal toxicology studies, we did not observe any CHIPS-related toxicologically significant changes in clinical observations, body weight, food consumption, haematology, coagulation, blood chemistry parameters, ophthalmoscopy, electrocardiograms, macroscopic or microscopic pathology or behaviour (full pre-clinical assessment disclosed in supplementary text 1). Notably, a transient decrease in mean arterial blood pressure (40%) was observed in beagles receiving a high dose of 20 mg $kg^{-1}$ CHIPS (supplementary text 1). However, mean arterial blood pressure returned to normal within 5 minutes post dosing. In all, these results suggest that side effects induced by CHIPS are unlikely to be observed in human subjects. As a result, the safety of CHIPS was subsequently studied in a set of six human subjects during a phase-1 clinical study.

Based on the toxicology studies, the administration of a single low dose of 0.1 mg $kg^{-1}$ CHIPS was considered safe and administered in 4 human subjects. First, we determined the presence of CHIPS in sera of the volunteers during different time-points post-CHIPS administration. In only two out of four subjects that received the CHIPS protein, subjects #104 and #105, CHIPS could be detected 15 min post-i.v. injection with a gradual decline after 1 hour (Figure 3b). CHIPS was not detected in the sera of subjects #103 and #106 (Figure 3b). These observed differences in the





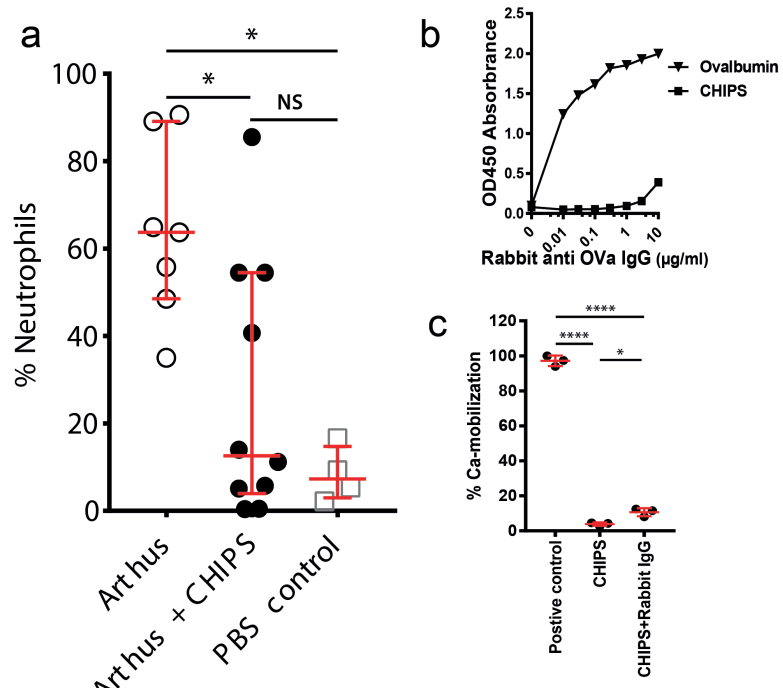


**Figure 2. CHIPS inhibits neutrophil migration *in vivo*.** (a) 60µg CHIPS (n=10) was injected i.p., and together with ovalbumin i.v. in hC5aRKI mice 30 minutes prior to inducing the Arthus reaction. Samples were compared to mice that did not receive CHIPS (n=7). Control mice (n=4) received PBS i.v. and i.p.. Peritoneal cavity lavage was performed 6-hours post Arthus induction. Percentage neutrophil influx was analysed by flow cytometry by gating on CD45+GR-1+F4/80- population and depicted as percentage of total leukocytes (CD45+) retrieved after peritoneal lavage. All groups consisted evenly out of female and male mice. Combined data from 2 independent experiments shown. (b) the presence of anti-OVA and anti-CHIPS antibodies in the rabbit anti-OVA IgG fraction was determined by ELISA. (c) To detect neutralizing anti-CHIPS antibodies in the rabbit anti-OVA IgG, CHIPS (500ng/ml) was incubated with 10µg/ml rabbit anti-OVA IgG or PBS. Subsequently, Fluo-4AM labelled human PMNs were incubated with CHIPS/Rabbit IgG or CHIPS/PBS and challenged with human C5a. Ca-mobilization was determined via flow cytometry and normalized to human PMNs that did not receive CHIPS. Significance was calculated using ANOVA, and when needed, followed by Kruskal-Wallis post-test for multiple comparison and displayed as *P<0.05,****P<0.0001 and NS for not significant.

detection of CHIPS in blood of the subjects seems to correlate with their initial level of anti-CHIPS antibodies. We hypothesized that the higher anti-CHIPS antibody titers hamper the detection of CHIPS by ELISA. Possibly, the epitope recognized by the capture monoclonal anti-CHIPS antibody is occupied by anti-CHIPS antibodies of the subjects. Consequently, we divided the 4 volunteers in 2 separate groups based on their anti-CHIPS antibody titer; anti-CHIPS low (subjects #104 and #105) and anti-

CHIPS High (subjects #103 and #106). The measured CHIPS serum concentration in subjects #104 and #105 are also potentially an underestimation due to the interference of pre-existing anti-CHIPS antibodies. In addition, for subjects #104 and #105 that had detectable levels of CHIPS 15 min post i.v. injection, CHIPS concentrations dropped a 2-log fold over the course of 24 hours (Figure 3b). These data show that CHIPS is taken up systemic within 15 min and cleared after 24 hours post i.v. administration. We calculated a predicted half-life of CHIPS to be at least 1.5 hours in humans.

CHIPS binds the C5aR1 on human neutrophils with high affinity *ex vivo* (26). However, *in vivo* binding of CHIPS could be hampered by circulating antibodies. In order to assess if CHIPS interacts with its therapeutic target, we determined the binding of CHIPS *in vivo* on neutrophils of the subjects. The amount of CHIPS present on the surface of neutrophils was determined at various time points post-CHIPS administration using a rabbit-anti-CHIPS antibody (36). Notably, the binding of CHIPS on the surface of neutrophils was only detected in subjects with a low anti-CHIPS antibody titer (subjects #104 and #105) (Figure 3c). It is possible that the anti-CHIPS antibodies present in serum also interfere with the direct detection of CHIPS on neutrophils. Therefore, the lack of a direct detection cannot exclude the absence nor presence of CHIPS bound to the receptor in the individuals with high anti-CHIPS antibody titers. All in all, we show that CHIPS binds circulating human blood neutrophils, confirming the interaction with target cells *in vivo*.

All tested subject had pre-existing anti-CHIPS antibodies. As a specific antibody response is mediated against CHIPS, it is likely that a re-challenge with CHIPS will lead to an increase in antibody titers. To determine the immunogenicity of CHIPS, anti-CHIPS serum titers were measured during different time points pre- and post-CHIPS administration. An increase in anti-CHIPS titer was observed in individuals receiving CHIPS that had a low anti-CHIPS antibody titer (subjects #104 and #105) pre-CHIPS administration (Figure 3d).The rapid boost of circulating IgG titers by the staphylococcal protein CHIPS in humans indicates high immunogenicity and pre-existing memory, supporting a concept of expected exposure to secreted staphylococcal proteins starting at early age (34, 35, 37).

## CHIPS induced adverse effects in humans

The administration of CHIPS in human subjects was tolerated by 2 subjects (subjects #103 and #104), moderately tolerated in subject #105 but subject #106 (subject with a high anti-CHIPS antibody titer) developed serious symptoms directly after the CHIPS infusion, which were diagnosed as an anaphylactic reaction (Supplementary text 2). No adverse events were reported in subjects receiving placebo. To determine whether the subjects developed a CHIPS-mediated inflammation response, white





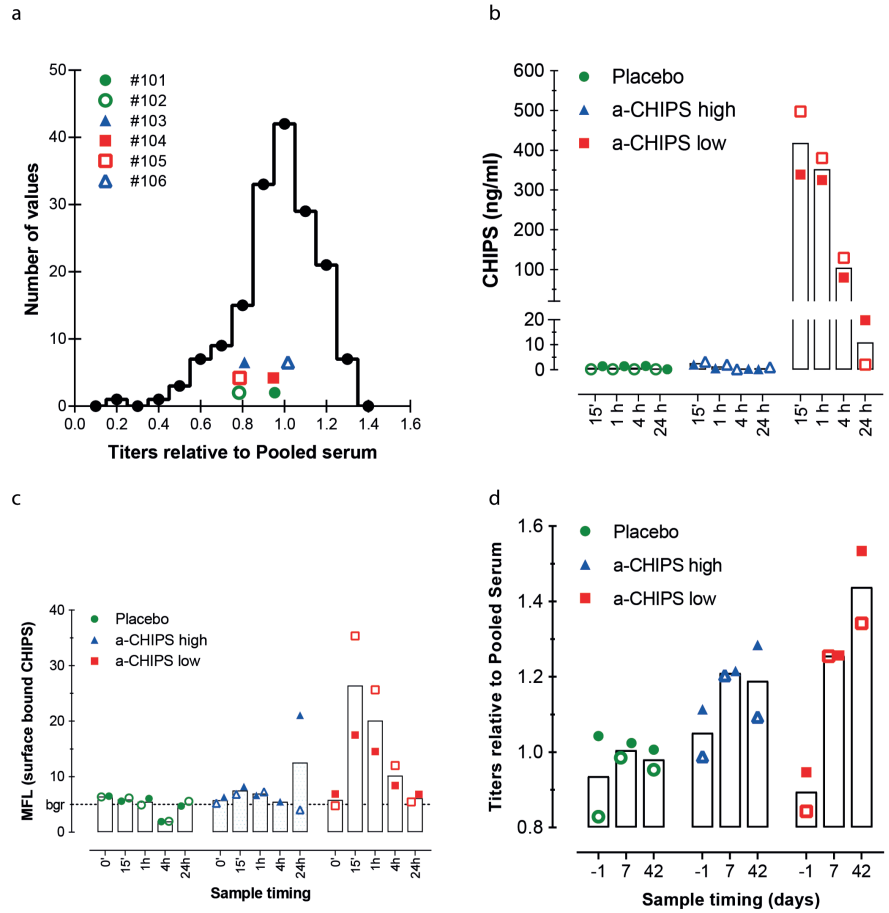


**Figure 3. CHIPS and anti-CHIPS antibodies in humans.** (a) Frequency distribution of IgG anti-CHIPS titer in healthy human donors (n=168). The titer was defined as the log dilution that gives an absorbance of OD0.300 after subtraction of background value. Titers were depicted relative to the mean human pooled serum (HPS) titer (3.75). Anti-CHIPS antibody titer of the 6 subjects before study entry are depicted in the same graph as comparison. The ■ represents subjects that had low anti-CHIPS antibodies (anti-CHIPS low), ▲ represents subjects with high anti-CHIPS antibodies (anti-CHIPS high) and the ● represents subjects in the placebo group. Open and closed symbols differentiate between receivers in each group. (b) Pharmacodynamics of CHIPS detected in the sera of the volunteers. CHIPS was measured by a specific capture ELISA at various time points after intravenous injection of CHIPS. (c) CHIPS is recovered on the surface of peripheral blood neutrophils. At various time points after i.v. injection, the presence of CHIPS bound to the surface of neutrophils was detected with rabbit-anti-CHIPS antibodies. Values are expressed as mean fluorescence (MFL) of gated neutrophils in EDTA whole blood samples. Background MFL value for the secondary FITC labelled conjugated was 6. (d) Immunogenicity of CHIPS in healthy human subjects. Specific IgG titer towards CHIPS were determined in all subjects before trial start, 7 and 42 days after trial closing and are depicted relative to HPS.

blood cell count (WBC) and C-reactive protein concentration (CRP) were measured pre- and post-dosing. CHIPS induced a transient leukocytopenia in the subjects receiving CHIPS that resolved within 2 days (Figure 4a). Within the group of subjects that received CHIPS there was a mild increase in CRP (average of 42 mg $ml^{-1}$) at day 2 post CHIPS dose compared to controls. CRP levels returned to normal when subjects were screened during follow up at day 15 (Figure 4b). This indicates that there was indeed an inflammation response upon CHIPS administration.

## Circulating immune complexes and increased serum tryptase

Mast cells play a central role in anaphylaxis and other allergic conditions. Immune complexes can activate mast cells by FcR crosslinking and through activation of complement and the generation of C5a (38). Circulating immune complexes (CIC) induce the abundant secretion of the serine proteinase tryptase by mast cells, which can be used as an indicator of anaphylaxis. Since all subjects had pre-existing anti-CHIPS antibodies, we evaluated whether intravenous administration of CHIPS leads to the formation of CIC. Circulating immune complexes were detected in the subjects receiving intravenous CHIPS (Figure 5a). Subject #106, who suffered an anaphylactic reaction following the administration of CHIPS, showed the highest CIC levels, contrary to subjects #104 and #105 who remained at baseline. CIC were also detected in subject #103, who has the highest anti-CHIPS antibody titer but reported only minor adverse effects. No CIC were detected in subjects that received the placebo.

Subsequently, we measured the serum tryptase levels in the subjects. An increase in serum tryptase concentration was detected in all subjects except subject #103, that reached a maximum at approximately 10 minutes post dose and continued to drop to baseline levels after 24h (Figure 5b). Notably, subject #106 had the highest levels of tryptase, which correlates with the high levels of CIC measured. These data suggest that CHIPS administration in subjects with high circulating anti-CHIPS titers results in an inflammatory response and adverse effects. Due to these effects, the study was stopped and no further administrations of CHIPS was undertaken.

Earlier studies showed that CHIPS does not bind other cells than those expressing the C5aR and there is no evidence of direct cell activation by CHIPS (27, 28, 33). The development of a second-generation CHIPS protein with a preserved activity but with reduced immunogenic properties could make a promising new candidate anti-inflammatory drug. Mapping of the epitopes for human IgG within the CHIPS protein will be an important first step in this development (39). We previously identified several unique conformational epitopes on CHIPS using affinity purified human IgG from a preparation for intravenous use (39). Despite developing a version of CHIPS with low interaction with pre-existing anti-CHIPS antibodies, the high immunogenicity of





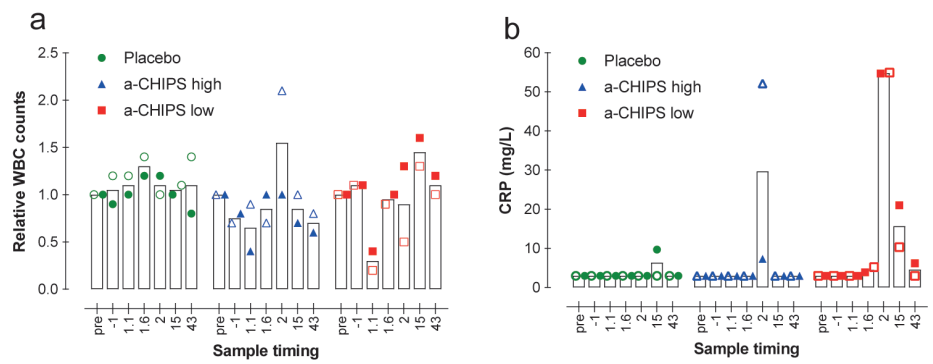


**Figure 4. CHIPS induces leukocytopenia and increased CRP levels in humans.** Levels of circulating (a) peripheral white blood cells and (b) serum inflammation marker CRP. At various time points after intravenous injection of CHIPS, WBC counts and CRP measurements were performed. (1.1 and 1.6 indicate 1 day and 1 or 6 hours respectively). Data for WBC are expressed relative to the value at T = 0 and data for CRP are expressed in mg/mL. The ■ represents subjects that had low anti-CHIPS antibodies (anti-CHIPS low), ▲ represents subjects with high anti-CHIPS antibodies (anti-CHIPS high) and the ● represents subjects in the placebo group. Open and closed symbols differentiate between receivers in each group.

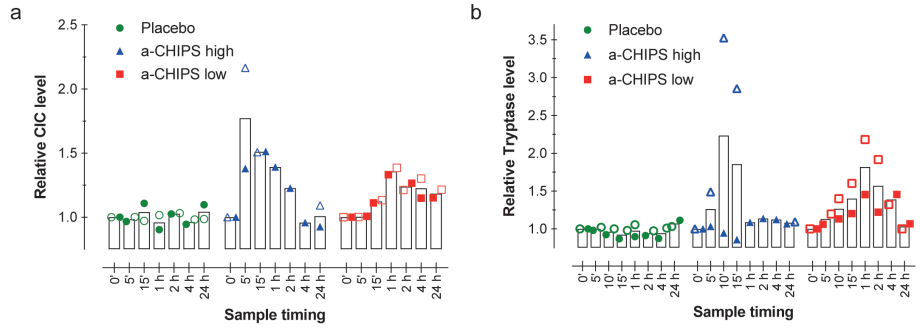


**Figure 5. Circulating immune complexes and increase serum tryptase.** Adverse effects of CHIPS as measured by levels of (a) Circulating Immune Complexes (CIC), and (b) mast cell marker tryptase. At various time points after intravenous injection of CHIPS, specific assays were performed for both markers. The ■ represents subjects that had low anti-CHIPS antibodies (anti-CHIPS low), ▲ represents subjects with high anti-CHIPS antibodies (anti-CHIPS high) and the ● represents subjects in the placebo group. Open and closed symbols differentiate between receivers in each group.

CHIPS will limit it suitable for therapies requiring a single administration. Despite the neutralizing effect of anti-CHIPS antibodies, we were able to detect significant serum concentrations of the CHIPS protein. The observation of the inhibition of this activity by anti-CHIPS antibodies together with the induction of an anaphylactic response shows that CHIPS in its current form is not suitable for use as an anti-inflammatory agent. Nevertheless, future molecules based on the CHIPS protein could very well be potential new candidates. Knowledge of the exact mechanisms of action and the active sites within the CHIPS molecule can lead to the development of small molecule anti-inflammatory drugs based on the CHIPS mechanism of action.

## Materials and Methods

### Ethics statement

The RCT study protocol (JPD-003/002/NL) and amendments were approved by an independent ethics committee. The study was performed in compliance with the 'Declaration of Helsinki' (Scotland, October 2000) and OECD Principles of Good Laboratory Practice and applicable regulatory regulations. For neutrophil isolation approval was obtained from the medical ethics committee of the University Medical Center Utrecht (METC-protocol 07-125/C approved March 01, 2010; Utrecht, The Netherlands). The use of animals was approved by the National Ethical Committee for Animal Experiments and performed according to the guidelines of the Central Animal Facility of the Utrecht University (Project# AVD115002016565).

### Isolation of Rabbit anti-ovalbumin IgG

IgG was purified from Rabbit anti-Chicken-Egg Albumin, delipidized whole antiserum (Sigma-Aldrich) using multiple runs over a 1 ml Protein-A HiTrap column (GE Healthcare Life Sciences) on an ÄKTA FPLC (GE Healthcare Life Sciences). Rabbit IgG was eluted from the column with 0.1 M citric acid, pH 3.0 and collected fractions were neutralized with 1M Tris-HCl, pooled and dialyzed against PBS. Protein concentration was determined at 280 nm using a molar extinction coefficient of 1.35 for Rabbit IgG.

### Peritoneal Arthus reaction and neutrophil migration

Human C5aR1$^{KI}$ mice were generated and characterized as described elsewhere (29) (40). The Arthus reaction was initiated upon i.v. injection in hC5aR1$^{KI}$ mice (male and female) of 100 µl of OVA (20 mg kg$^{-1}$ of body weight; Sigma-Aldrich) immediately followed by an i.p. injection of 800 µg of rabbit anti-OVA IgG (Sigma) in 500 µl PBS. For mice in the CHIPS group, 60 µg CHIPS was administered i.p. 30 minutes prior to initiation of the Arthus reaction and simultaneously with OVA i.v.. For the control





group, PBS was administered i.v. and i.p.. Mice were euthanized by $CO_2$ suffocation 6-hours after the onset of the peritoneal Arthus reaction and the peritoneal cavity washed with two times 5 ml of ice-cold RPMI 0.1% HSA/5mM EDTA. Peritoneal fluid was recovered and centrifuged at 1200 rpm for 10 min to collect the exudate cells. Cell pellets were resuspended in 500 µl buffer and counted with trypan blue in a TC20 automated cell counter (BioRad). Cells were stained in the presence of a Fcγ-receptor blocker, with anti-mouse CD45-APC (clone 30-F11, BD Biosciences), anti-mouse Gr1-PE (1A8, BD Biosciences), anti-mouse F4/80 FITC (BM8, eBioscience), anti-human C5aR-FITC (clone S5/1, SeroTec), isotype rat-IgG2a-FITC (R&D) and rat-IgG2b-PE (BD Biosciences). Samples were analyzed by flow cytometry. Collected peritoneal cells were washed with PBS and the cell number adjusted to $5\times10^6$ cell ml$^{-1}$. Cytospin slides were prepared with 50 µl $5\times10^4$ cell suspension and stained with DiffQuick. The percentage of neutrophils was determined by flow cytometry analysis and confirmed by the number of neutrophils based on morphology following DiffQuick staining. Mouse neutrophils were isolated from bone-marrow as described elsewhere (41, 42). Briefly, bone marrow cells were collected by flushing the femurs and tibias with 10 ml of cold HBSS + 15 mM EDTA + 30 mM Hepes + 0.1 % HSA. A two-layer Percoll density gradient (2 ml each in PBS) composed of 81% and 62.5% was used to enrich neutrophils from the total leucocyte population. Interphase between 62.5% and 81% was collected. Cells were washed once with buffer and resuspended in PRMI1640 with 0.1% HSA. Staining of bone marrow cells was performed as described above.

### Preclinical assessment of CHIPS toxicity in animal models

Conventional pre-clinical toxicology studies were preformed to investigate the safety of intravenous CHIPS. These included; (I)The effects of CHIPS on various cardiovascular and respiratory parameters in one group of three anesthetized beagle dogs. The dogs were administered CHIPS in incremental doses of 0.2, 2.0 and 20 mg kg$^{-1}$ , infused intravenously over 1 minute at approximately 30 minute intervals. (II) Behavioral ('Irwin') test in mice: CHIPS was administered as a single intravenous injection to male ICR CD-1 mice (3 per group) at doses of 7.5, 25 and 75 mg kg$^{-1}$ in order to assess effects on general behavior. An additional group received an equivalent volume (10 ml kg$^{-1}$) of vehicle (0.9% w/v sterile saline). (III) Acute intravenous toxicity study in rats: Intravenous administration of 96.1 mg·kg$^{-1}$ CHIPS as a single dose (the maximum practically achievable due to volume considerations) to 5 male and 5 female rats. (IV) Acute intravenous toxicity in mice: Intravenous administration of 96.1 mg kg$^{-1}$ CHIPS as a single dose to 5 male and 5 female mice. (V) Seven day intravenous bolus preliminary toxicity study in rats (24 males and 24 females, maximum dose 10 mg kg$^{-1}$). (VI) Seven day intravenous

bolus toxicity study in rats (76 males and 76 females, maximum dose 10 mg $kg^{-1}$). (VII) Seven day intravenous bolus dose range finding study in dogs (2 males and 2 females, maximum dose 20 mg $kg^{-1}$). (VIII) Seven day intravenous bolus toxicity study in the dogs (12 males and 12 females, maximum dose 20 mg $kg^{-1}$).

### Inclusion of human volunteers

Full description of study population, including number of subjects, inclusion, exclusion and removal criteria are described in supplementary Protocol No.: JPD-003/002/NL. Briefly, inclusion criteria for healthy volunteers were as follows: (I) Adult males within an (II) age range 18-50 and (III) a body mass index (BMI) of 18-30 kg $m^{-2}$. Medical screening was divided in 2 parts. Subjects were screened for anti-CHIPS antibody titers. Only subjects with a low titer (equal or lower to 3.92, defined as the log of the serum dilution that gives an absorbance value of 0.300 in the ELISA) were screened for the second part within 3 weeks before dosing and include: medical history, physical examination, measurement of blood pressure, heart rate, respiration and temperature, alcohol breath test, blood and urine tests, electrocardiogram (ECG) and drug screening.

### Admission and follow up

Full description of the admission and follow up, treatments and stopping rules are described in (supplementary Protocol No.: JPD-003/002/NL). Briefly, six selected subjects (4 receiving CHIPS and 2 controls) were admitted to the Clinical Pharmacology Unit (Kendle, Utrecht, The Netherlands) on the day before dosing. Baseline measurements, including blood samples for safety, urinalysis, interim medical history, physical examination, vital signs and ECG were done. On the day of dosing CHIPS (0.1 mg $kg^{-1}$ administered as a single dose of sterile frozen isotonic saline solution containing CHIPS at a concentration of 5 mg $ml^{-1}$) or placebo (0.9% NaCl) was administered by intravenous infusions over 5 minutes. Subjects were connected to a telemetry system for cardiac monitoring from 30 minutes before dosing until 4 hours after start of dosing. The blood pressure of subjects was measured continuously using a Finapres from 5 minutes before dosing until 30 minutes after start dosing. Vital signs were measured and ECG's were made at certain time points during the admission period. For safety, clinical status and laboratory values (haematology, biochemistry, coagulation and urinalysis) of all subjects were monitored. Adverse events were documented and characterized according to their severity and relationship to CHIPS or placebo. The subjects were discharged at 24 hours after dosing. Two weeks after dosing subjects returned to the Unit for a visit to evaluate vital signs, ECG, blood and urine and anti-CHIPS antibody level. A follow up visit was scheduled 6 weeks after dosing.





### Cloning and expression of CHIPS

CHIPS was cloned and expressed as described earlier (26, 36). Briefly, the CHIPS gene (*chp*; GenBank: AF285146.1), without the signal sequence, was cloned into the pRSET vector directly downstream the enterokinase cleavage site and before the EcoRI restriction site by overlap extension PCR. Bacteria were lysed with CelLytic B Bacterial Cell Lysis/Extraction Reagent (Sigma) and lysozyme according to the manufacturer's description. The histidine-tagged protein was purified using a nickel column (HiTrap Chelating HP, 5ml, Amersham Biosciences) following the manufacturer's instructions and cleaved afterwards with enterokinase (Invitrogen). Samples were checked for purity and presence of protein using 15% SDS-PAGE (Polyacrylamide gel electrophoresis, Mini Protean 3 System, Bio-Rad) and Coomassie Brilliant Blue (Merck) staining.

### Purification of CHIPS for intravenous use

Full-length CHIPS was expressed in *E. coli* containing the coding sequence of CHIPS directly downstream to PelB coding sequence in a growth media consisting of soya peptone and yeast extract in 8 liter fermentation media. CHIPS was isolated both from the growth media and the cells by a two stage cation exchange purification process followed by a desalting step. The bacterial cell pellet was resuspended in phosphate buffer (30 mM; pH 7.0), containing NaCl (10 mM), DTT (10 mM) and frozen. This was subsequently thawed at 37°C, incubated on ice and sonicated. After centrifugation at 15,000 rpm, an amber colored 'cell' supernatant was recovered. The supernatant was diluted four-fold with 30 mM phosphate buffer and passed over a Source S-30 column. The material was eluted with a phosphate buffer salt gradient and fractions containing CHIPS were combined and purified further by using a polishing column with a shallow salt gradient. Fractions containing CHIPS with purity greater than 97% (by HPLC) were combined and passed through a Sephadex G 25 desalting column to remove phosphate and excess of sodium chloride. Endotoxin was removed by gently shaking over resin (Biorad) and the preparation was sterilized through ultra-filtration. We confirmed the purity by HPLC-MS on a Microbondapac CN-RP column with a mobile gradient phase consisting of water-TFA to Methanol-TFA. The end product was diluted with sterile saline to the desired concentration and stored at -20°C.

### Isolation of human PMN

Blood obtained from healthy volunteers was collected into tubes containing sodium heparin (Greiner Bio-One) as anticoagulant. Heparinized blood was diluted 1/1 (v/v) with PBS and layered onto a gradient of 10 ml Ficoll (Amersham Biosciences, Uppsala, Sweden) and 12 ml Histopaque (density 1.119 g $ml^{-1}$; Sigma-Aldrich, St.

Louis,MO). After centrifugation (320 g, for 20 min at 22°C), the neutrophils were collected from the Histopaque phase and washed with cold RPMI 1640 medium containing 25 mM HEPES buffer, L-glutamine (Invitrogen Life Technologies) and 0.05% HSA (Sanquin, Amsterdam, the Netherlands). The remaining erythrocytes were lysed for 30 s with ice-cold water, after which concentrated PBS (10 x PBS) was added to restore isotonicity. After washing, cells were counted and resuspended in RPMI-1640 / 0.05% HSA at $10^7$ neutrophils $ml^{-1}$.

### Determining Circulating Immune Complexes, C-Reactive Protein and serum tryptase

CIC were determined by 2 different ELISA's from Quidel (San Diego, CA): the CIC-C1q enzyme immunoassay is based on the principle that complement fixing IC will bind to immobilized human C1q purified protein; the CIC-Raji Cell Replacement enzyme immunoassay measures IC containing C3 activation fragments by using a mAb that specifically binds the iC3b, C3dg and C3d activation fragments of C3 in a manner which is analogous to the classical Raji cell CR2 binding reaction. The data of both assays were combined and results expressed relative to the value at time point 0. CRP levels were determined by the diagnostic department according to standard protocols. Serum derived tryptase (both α-and β-form) was measured on the UniCAP®-100 using the ImmunoCAPTM technology (Pharmacia Diagnostics, Woerden, The Netherlands). The normal geometric mean for serum tryptase in healthy controls is 5.6 $\mu g\ l^{-1}$. Results were expressed relative to the value at time point 0.

### ELISA for anti-CHIPS antibodies and CHIPS levels

Rabbits were immunized with recombinant CHIPS using Freund's Complete Adjuvants and boosted with Freund's incomplete adjuvants. Bleedings were checked for reactivity with CHIPS by ELISA as described for human anti-CHIPS antibodies (see below). From the final bleeding, IgG was purified by standard Protein-G (Pharmacia) affinity chromatography according to the manufacturer's instructions. For the anti-CHIPS ELISA, microtiter plates (Greiner) were coated with 50 µL CHIPS per well at 1 $\mu g\ ml^{-1}$ in PBS overnight at 4°C. All wash steps were performed thrice with PBS-0.05%Tween-20 and subsequent incubations were done for 1 hour at 37°C. Plates were blocked with PBS-0.05%Tween-20 4% BSA, washed and incubated with sera or antibodies diluted in PBS-0.05%Tween-20 1% BSA. Bound antibodies were detected with species-specific goat anti-IgG conjugated with peroxidase (all from Southern, Birmingham, USA) and TMB as substrate. The reaction was stopped with $H_2SO_4$ and the absorbance measured at 450nm in a BioRad ELISA-reader. For the capture ELISA, microtiter plates were coated with 50 µL α-CHIPS mAb 2G8 at 3 µg $mL^{-1}$ in PBS overnight at 4°C. Plates were blocked with PBS-0.05%Tween-20 4% BSA, washed and incubated with diluted samples and a two-fold dilution range of CHIPS as standard in PBS-0.05%Tween-20 4% BSA. Subsequently, plates were incubated with 0.33 µg $mL^{-1}$ rabbit α-CHIPS IgG and 1:5000 diluted peroxidase-conjugated goat anti-rabbit IgG (Southern). Bound antibodies were quantified with TMB as substrate, the reaction stopped with 1 N $H_2SO_4$ and OD was measured at 450 nm on a BioRad ELISA reader.





### Statistical analysis

Calculations of statistical analyses were performed using Prism 7.0 (GraphPad Software). Flow cytometric analyses were performed with FlowJo (Tree Star Software). Significance was calculated using analysis of variance (ANOVA) followed by Kruskal-Wallis as post-test correction for multiple comparison. All statistical methods with regards to the human trails are described in the supplementary (Protocol No.: JPD-003/002/NL.)

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## Supplementary Text 1: Pre-clinical assessment of CHIPS as a therapeutic agent

CHIPS successfully dampened the C5aR dependent Arthus reaction in a mouse model expressing the hC5aR . To allow testing of CHIPS in human volunteers, pre-clinical safety experiments in non0hman subjects were required. In none of the toxicology animal studies did administration of CHIPS cause any CHIPS related toxicologically significant changes in clinical observations, body weight, food consumption, haematology, coagulation, blood chemistry parameters, ophthalmoscopy, electrocardiograms, macroscopic or microscopic pathology or behavior. The effects of CHIPS on various cardiovascular and respiratory parameters in anesthetized beagle dogs was examined. In the dogs receiving low dose CHIPS (0.02 and 2 mg·kg-1) there was no evidence of cardiovascular or respiratory effects when compared to infusion of vehicle (isotonic saline). Following intravenous administration of 20 mg·kg-1 CHIPS a transient decrease in mean arterial blood pressure (40%) was recorded approximately 1 minute after start of administration.. Mean arterial blood pressure levels returned to pre-dose levels within approximately 5 minutes following the start of dosing. The effect on blood pressure coincided with transient, inconsistent changes in heart rate. One dog was administered a repeat intravenous dose of CHIPS (20 mg·kg-1) approximately 30 minutes following the first administration of CHIPS. Transient effects on cardiorespiratory parameters similar to those recorded following the first dose were not apparent after the repeat administration of CHIPS. However, the second administration produced a prolonged reduction in mean arterial blood pressure, reaching a maximum of 18% at approximately 30 minutes following the second administration. In this animal only, twelve minutes following the repeated administration of CHIPS a generalized skin reaction appeared consistent with some form of mild allergic reaction. The results of this study suggested that cardiorespiratory effects are unlikely to be observed in the human subjects in the used dose range (0.1 mg·kg-1). Furthermore, any effects that might occur were expected to be transient and reversible.

CHAPTER 6

# General discussion

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# General discussion

During infection, our innate immune system is the first line of defense and has evolved to clear invading bacteria immediately. Neutrophils are an important component of the innate immune system and provide a front line of defense against bacterial infection (1, 2). Immune receptors play a vital role to generate, shape and modulate neutrophil activity, effector functions and responses to microbes (3, 4). However, "*everything is a double-edged sword*". Neutrophils do not always act in ways beneficial to the host: uncontrolled neutrophil responses can exacerbate and even cause autoimmune and inflammatory diseases (5, 6). Therefore, neutrophils need to be tightly regulated in order to respond quickly to different invading bacteria and to prevent the host from suffering excessive and damaging immune responses. This is also true for bacteria. Bacteria are all around us and only some of them are responsible for a large number of human infection disease, while others play a key role in human necessary functions such as digestion. *Commensalism*, is a concept developed by Pierre-Joseph van Beneden (1809-1894), where microbiota colonize a host in a non-harmful coexistence. Most commensal bacteria reside on epithelial surfaces that come in contact with the external environment. They are commonly found on the skin, as well as in the respiratory tract and the gastrointestinal tract. Commensal bacteria acquire nutrients, and a place to live and grow, from their host. In some instances, commensal bacteria may become pathogenic and cause disease, or they may provide a benefit for the host. To better colonize and to survive in more hostile environments, such as inside the human body, pathogenic bacteria have evolved various immune interaction molecules to evade neutrophil attacks. Thus, this is a complex molecular battel between neutrophil and bacteria.

The aim of this thesis was to develop novel strategies to prevent or treat infectious or inflammatory diseases. A better understanding of innate immune responses and host-pathogen interactions is critical for this development. The focus of this thesis was to characterize the role of immune receptors in neutrophil biology and to identify new bacterial immune modulatory molecules.

### Why do neutrophils utilize so many receptors?

Neutrophils play an indispensable role in the host defense against bacteria, but have also been shown to be important in other inflammatory diseases such as autoimmunity and cancer. As described in **Chapter 1,** neutrophils are equipped with a large amount of immune modulating receptors that have the abilities to achieve their effector functions by activation, suppression or both. It is interesting to ask why neutrophils need to utilize such a large amount of receptors? It is most likely that neutrophils must recognize and respond to many different internal and external





environmental conditions that inform the neutrophil to remain resting, to become ready for combat or to destroy invading microbes.

Neutrophils utilize a diverse range of surface receptors for recognizing microbial infection and for maintaining neutrophil homeostatic. The different immune modulating receptors can be grouped into activatory and inhibitory immunoreceptors based on receptors that have the potential to enhance or suppress neutrophil effector functions respectively. Binding of ligands to activatory immunoreceptors including Fc receptors (FcγRI and FcγRIIa and FcεRI), G protein-coupled receptors (GPCRs), cytokine and chemokine receptors, adhesion receptors (e.g. integrins or selectins) and pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) or C-type lectin receptors (CLRs) (4, 7, 8), induces neutrophil activation and effector function such as phagocytosis, ROS production, NET-formation, degranulation and cytokine release. In contrast, binding of ligands to inhibitory receptors including FcγRIIb, leukocyte-associated Ig-like receptor 1 (LAIR-1) and signal inhibitory receptor on leukocytes 1 (SIRL-1), induces suppression of neutrophils effector functions. Remarkably, unlike the other activatory receptor families, FcRs family contain both activatory and inhibitory receptors, with structures containing Ig-like binding domains that mediate signaling via intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) and immunoreceptor tyrosine-based inhibitory motifs (ITIMs) respectively. Upon ligand binding to ITAM-containing activatory receptors such as FcγRI and FcγRIIa and FcεRI, the ITAM become phosphorylated and this leads to recruitment of Syk tyrosine kinase, activation of Syk, several tyrosine kinase substrates and subsequent downstream signaling (9). Upon ligand binding to ITIM-containing inhibitory receptors, such as FcγRIIb, LAIR-1 and SIRL-1, ITIM becomes phosphorylated, leading to docking of SH2 domain containing tyrosine phosphatases such as SHIP-1, Shp1 and Shp2, and suppression of activatory downstream signaling (3, 4). Consequently, cross-talk of activatory and inhibitory receptors are important to balance host immune responses. Therefore, in order to better understand why neutrophils express such a large diversity of immunoreceptors, one needs to know where and when the receptor is expressed and its functions in both homeostatic and the relevant pathologic conditions. In particular, knowledge of those receptors expressed on neutrophils where there is no or limited knowledge of their functions and biological ligands is required. This includes receptors of the leukocyte immunoglobulin-like receptors (LILR) family that are immunomodulatory receptors expressed on a variety of immune cells (10, 11). LILR can divided into activatory (LILRA) and inhibitory (LILRB) by containing two different cytoplasmic region. Consequently, binding of ligands to LILRA or LILRB is hypothesized to lower or enhance the immune activation threshold. Studies have shown that some LILRs can modulate neutrophil activation (12, 13), while, robust knowledge of expression and functions of all LILRs on neutrophils is lacking.

In **Chapter 2**, we illustrated that LILRA2, LILRA6, LILRB3 and LILRB4, were expressed at the surface of resting neutrophils and LILRB2 was expressed in the granules. Expression of the rest of LILRs was not assessed on the neutrophils due to the lack of available monoclonal antibodies. Furthermore, we characterized that LILRB impairs FcγR-mediated neutrophil effector functions including ROS production, phagocytosis and killing of microbes, but we did not identify immunomodulatory effects from LILRA. Emerging evidence indicates that some bacteria directly interact with LILR. Specifically, *Staphylococcus aureus*, *Escherichia coli* and *Helicobacter pylori* bind LILRB1 and LILRB3 (14), whilst *Mycobacterium spp* recognize LILRA1 and LILRB5 (15). Since these bacteria are both commensals and opportunistic pathogens and the bacterial ligands remains unknown, it is unclear whether neutrophils have evolved to express LILR to balance the immune reaction during the bacterial infection or whether bacteria have evolved to utilize LILR to prevent neutrophils killing. Therefore, identification of the biological ligands of LILR will improve knowledge of the physiological role of LILRs in neutrophil biology. We were able to produce LILR overexpression cell lines and LILRCD3ζ reporter cell lines, this could not only help us to know which LILR(s) is/are more responsible during different bacterial infections, but also may help us to identify the biological ligands of LILR in the future.

### Expanding the repertoire of bacterial immune interaction molecules via Phage display

In the arms race of host-microbe co-evolution, many bacterial species, whether primary pathogens or commensal bacterium, have evolved various ingenious ways to evade host immune responses. This includes expression of a large amount of immune interaction molecules. *S. aureus*, as one of the most well studied bacteria, secretes a huge repertoire of immune interaction molecules that can manipulate immune response. Remarkably, most of those molecules are secreted proteins. A closer look at the genome of *S. aureus* indicates that up to 270 proteins are secreted. So far, over 35 staphylococcal immune interaction molecules have been described (16). Thus, we believe there are more proteins that need to be identified and characterized. Furthermore, many of the *S. aureus* immune evasion molecules have multiple functions, so it is likely that new functions for known molecules could be described in the future. Worldwide, *S. aureus* remains a prominent cause of bacterial infections in human and has become increasingly resistant to antibiotics, including both hospital-acquired and community-acquired MRSA. Therefore, to expand the repertoire of immune interaction molecules of *S. aureus* and to characterize new functions of existing immune evasion molecules will lead to the discovery of appropriate therapeutic targets to fight this smart bacterium.





In **Chapter 3**, we set out to identify new *S. aureus* proteins that interact with human neutrophils using a *S. aureus* secretome phage display library. In combination with Whole genome Illumina sequencing, Staphylococcal superantigen-like protein 13 (SSL13) was identified to bind human neutrophils. SSL13 belongs to a family of 14 secreted proteins which were previously demonstrated to modulate immune evasion (16–18). In contrast to other characterized SSLs, SSL13 was characterized as neutrophil activator via binding to formyl peptide receptor 2 (FPR2). The FPR2 is expressed by human neutrophils and SSL13 interaction leads to neutrophil activation and chemotaxis. Phenol-soluble modulins (PSMs) are small peptides that are also secreted by *S. aureus*, which are known to activate and attract both human and mouse neutrophils via FPR2 (19–21). In comparison to SSL13, PSMs have a completely different structure. In addition to this, micromolar concentrations of PSM have cell lytic activity which is independent from FPR2. A previous study revealed that PSMs only function intracellularly, since serum can fully block PSMs functions in both the cell lysis and FPR2-mediated neutrophil activation (21). However, SSL13 activity was not inhibited by serum and is not cytotoxic for neutrophils. Moreover, in contrast to PSMs, SSL13 showed a high degree of human specificity and was not able to efficiently activate mouse neutrophils. Another difference is the regulation and expression of PSMs, which was shown to be induced after uptake by neutrophils (21). Where and when SSL13 is expressed still remains unclear. We were unable to show expression of SSL13 neither under standard bacterial cell culture nor after uptake by neutrophils. However, this also could be that *S. aureus* has evolved to not express, or to express low levels of SSL13, in order to prevent priming neutrophils via FPR2.

In contrast to the majority of the SSLs (1-11) that are located on SPI-2, SSL13 together with SSL12 and SSL14 are located on IEC-2 cluster. It is interesting that FLIPr or its homologue FLIPr-like (FLIPrL) are located on the same IEC-2 cluster as SSL13. FLIPr and FLIPrL bind and inhibit FPR2 signaling function (22, 23), and this may serve to counteract FPR2 activation through SSL13. Collectively, this may contribute to the ability of *S. aureus* to adjust a favorable balance between neutrophil activation and inhibition.

There are still many questions that need to be addressed, such as whether SSL13 has a yet unknown additional function, may be in combination with the co-expressed SSL12 and SSL14, since SSL12-13-14 share a single promotor. Why does *S. aureus* secrete a neutrophil alarming protein? How does *S. aureus* benefit from this? All these questions need be examined in the future.

From studying *S. aureus*, we have learnt a lot about commensals and opportunistic bacteria. We are also interested in better understanding other commensal bacteria, especially the biggest commensal community in the gut. There are trillions of microbial organisms colonized in the human gut that form the gut microbiota.

A large amount of studies have shown that the gut microbiota play an essential role in maintaining homeostasis and have a profound effect on the development and maturation of the immune system. Changing the composition of the gut microbiota has been associated with many non-infectious diseases including inflammatory bowel diseases, autoimmune diseases, cancer and diabetes (24–26). What we want to ask is why commensal can be commensal? How can efficient commensal bacteria evade host immune responses and successfully colonize the gut? Do these commensals also utilize some immune interaction molecules to favorably balance host immune response? Our hypothesis is that gut microbiota, like *S. aureus,* may secrete different immune interacting molecules that can modify host immune responses and provide balance between pro-inflammatory and regulatory responses (27). By this they can keep the gut symbiotic environment. How can we fish out these immune interacting molecules from gut microbiota? Most of the gut bacteria species are anaerobic bacteria, and they are difficult to culture under normal lab conditions. Additionally, it is also a laborious work to express and study proteins from each individual gut bacteria separately. Instead, high-throughput approaches are required. We have demonstrated that secretome phage display is a powerful technique to identify immune evasion proteins from multiple organisms interacting with immune components. No live bacteria are required for making phage libraries except *E. coli*. All these properties of phage display technique provide a useful tool to study our hypothesis.

We constructed a phage display library of the human intestinal microbiome by using feces from healthy volunteers. Using various targets, including immune components such as complement factors and antimicrobial peptide or more complex targets such as cells and intestinal organoids, for phage selection we identified 7 bacterial proteins interacting with key components of the host intestinal immune system. As described in the **Chapter 4**, following selection on complement components we identified a protein with repeating domains from multiple intestinal bacteria as a novel immunoglobulin binding molecule. We named this protein as ProteinR. ProteinR reveals a broad binding ability to different immunoglobulins from different species and also to different human immunoglobulin subtypes. In comparison to other well characterized bacterial immunoglobulin binding proteins such as Protein A (Spa) from *S. aureus*, Protein G from group G streptococci and protein L from some strains of *Peptostreptococcus magnus*, ProteinR binds to Igs via F(ab')2 independently of the variable region of the Fab heavy chain ($V_H$). But ProteinR interferes with antibody recognizing its antigen, furthermore it inhibits antibody mediated neutrophil phagocytosis. We also found that ProteinR-antibody complexes are able to trigger Fc-receptor mediated neutrophil ROS production. However, to elucidate what is the actual function of





immunoglobulin binding in the gut, further studies need to be performed. We have two hypotheses about the potential function of ProteinR in the gut system. Firstly, we hypothesis that gut commensal bacteria evolved ProteinR to better colonize the gut epithelial surface. Secretory Igs such as sIgA, IgM, and IgG that are present in mucosal surfaces potentially provide a first line of defense against microorganisms (28). Secretory IgA is produced with a large amount everyday by humans to play a key role in preventing bacterial adherence to the gastrointestinal surfaces (29). However, sIgA function needs to be balanced, otherwise it also will damage good bacterial adherence. To test this hypothesis, future studies should perform bacterial adherence assays using gut epithelial cell lines or on the human organoids. The second idea is related to the ability of ProteinR-antibody complexes to trigger neutrophil ROS production. Previous studies have shown that gut commensals can induce ROS on epithelial cells and that they can modulate intestinal epithelial cell pro-inflammatory responses. However, these studies have not shown how commensal bacteria induced ROS production (30). Do ProteinR-antibody complexes also trigger a response in intestinal epithelial cells? If so, do commensal bacteria also use this strategy to induce immune tolerance that ensures their survival within the host without endangering it. All these questions need further studies to provide answers.

## Immune evasion molecules: From virulence to therapy

To summarize, studying host-pathogen interactions, we need to improve characterization of the role of immune receptors in neutrophil biology (**Chapter 2**) and to expand the full repertoire of bacterial immune evasion mechanisms (**Chapter 3** and **Chapter 4**). Moreover, understanding whether these mechanisms and interactions can be targeted or exploited in future therapeutics is required in order to prevent or treat infection or inflammation.

In **Chapter 1**, we introduced different immune-receptors on neutrophils. An important receptor is the C5a-receptor 1(C5aR1), a receptor expressed on a wide variety of inflammatory cells that belongs to the family of seven-transmembrane G-protein coupled receptors (GPCR). The C5aR1 plays an important role in recognizing invade bacteria and in activating neutrophil effector functions. However, it is also shown that the C5aR1 is also involved in a variety of other inflammatory processes such as ischemia-reperfusion injury, rheumatoid arthritis, asthma, immune complex diseases, neurodegeneration and Alzheimer's disease (31–33) (32). Targeting of C5aR1 has also shown to be beneficial in some of these disease processes in animals. Furthermore, inhibiting the proteolytic cleavage of C5 to C5a with Eculizumab, a monoclonal anti-C5 antibody, is used for the treatment of paroxysmal nocturnal haemoglobinuria (PNH), further emphasizing the relevance

of the C5aR1 as a therapeutic target. Chemotaxis Inhibitory Protein of *S. aureus* (CHIPS) binds to the N-terminus of human C5aR1 with high affinity (Kd=1.1nM) and functionally blocks the interaction with C5a, thus preventing activation and antagonizing chemotaxis. In **Chapter 5**, we use the immune complex-mediated Arthus reaction model that results in an inflammatory response and neutrophil recruitment that is mainly C5a mediated. We show that CHIPS can inhibit hC5aR1 mediated neutrophil migration in hC5aRKI mice *in vivo*. This indicates the therapeutic potential of CHIPS as a C5aR1 inhibitor *in vivo*. However, administration of CHIPS in human subjects led to immune complex formation with circulating anti-CHIPS antibodies and induced adverse effects that dampen the use of CHIPS in its current form as a therapeutic candidate. Nevertheless, future development of small homologue molecule anti-inflammatory drugs based on bacterial immune interaction molecules could very well be potential new candidates.

## Concluding remarks

The research described in this thesis illustrates the interactions between neutrophils and bacteria. In our studies we have characterized the role of immune receptors in neutrophil biology and expanded repertoire of bacterial immune interaction molecules and whether these mechanisms and interactions can be targeted or exploited in future therapeutics. We believe that tying neutrophils and bacteria together will provided new intervention strategies for both infectious and inflammatory diseases.

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## CHAPTER 7

# &

**English summary**

**Nederlandse samenvatting**

**Acknowledgements**

**Curriculum Vitae**

**List of publications**

# English summary

The human body is a constantly changing ecosystem comprised of trillions of microbial organisms collectively known as the microbiome. In health, human keep a symbiosis relationship with these microorganisms, but once this balance is destroyed, the symbiotic bacteria will be transformed into pathogenic bacteria, causing local and even systemic infection disease. Especially, due to abusing of antibiotics, some of commensal bacteria, also known as "super bug", becoming increasingly virulent and resistant to antibiotics. Infections caused by multidrug-resistant bacteria represent a major public health burden. Once bacteria bacteria invade their host, neutrophils (also known as PMN [polymorphonuclear leukocytes]), as the most abundant phagocytic cell in the human blood, are the first cell arrived at infection place and provide the front line of host defense against invading microbes. Neutrophils play a crucial role in protecting the host from bacterial infections and also have a pivotal role in orchestrating inflammation.

This thesis is primarily focused on studying neutrophil-pathogen interactions, and then translating this this information to develop therapeutic strategies for infectious and inflammatory diseases. In **chapter 1**, we first describe the role of neutrophils in the fight against the bacterial infection. We then highlight the different bacterial secreted "immune evasion" strategies that enhance resistance to neutrophil attacks. Thirdly, we emphasize future research perspectives to improve knowledge of neutrophil function & bacterial pathogenesis.

Neutrophils utilize a diverse range of surface receptors for recognizing microbial infection and for maintaining neutrophil homeostatic. Upon stimulation, if neutrophil responses are too weak, then hosts are susceptible to infection, for example neutropenic cancer patients often acquire *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) infections. However, if neutrophil responses are too strong, then this can damage the host and such responses are associated with the morbidity of many inflammatory diseases, and these include arthritic diseases, COPD and cardiovascular diseases. We therefore need new approaches to re-balance neutrophil responses. We know that neutrophils are regulated by groups of immune receptors, some of which stimulate cell activity and some of which inhibit cell activity. Its likely that there are 100s of different receptors expressed on neutrophils, and whilst the biology of some receptors is well characterized, basic knowledge of certain receptors in neutrophil biology is neglected. This means we do not appreciate the full repertoire of strategies that could be used to modulate neutrophils. Studies have shown that some leukocyte immunoglobulin-like receptors (LILRs) can modulate neutrophil activation while, robust knowledge of expression and functions of all LILRs on neutrophils is lacking. In **chapter 2**, we





illustrated that LILRA2, LILRA6, LILRB3 and LILRB4, were expressed at the surface of resting neutrophils and LILRB2 was expressed in the granules. We further characterized that LILRs modulate FcγR-mediated neutrophil effector functions including ROS production, phagocytosis and killing of microbes. We were able to generate LILR overexpression cell lines and LILRCD3ζ receptor cell lines, this could not only help us to know which LILR(s) is/are more responsible during different bacterial infections, but also may help us to identify the biological ligands of LILR in the future.

*S. aureus* is one of the most common cause of infection in both hospitals and the community. In addition, to better colonize and to survive in more hostile environments, such as inside the human body, *S. aureus* have evolved various immune interaction molecules to evade immune system. In **chapter 3**, we set up a high-throughput binding selection strategy, which acts as a novel approach to identify new *S. aureus* proteins that interact with human neutrophils. In contrast to many other immune evasion proteins that inhibit immune responses, we identified staphylococcus superantigen like protein 13(SSL13), one of a family of 14 secreted proteins which were previously demonstrated to modulate immune evasion, as a chemoattractant and a neutrophil activator that acts via the FPR2. Therefore, SSL13 is a unique SSL member that does not belong to the immune evasion class, but is a pathogen alarming molecule. Our study provides a new concept of SSLs; SSLs not only inhibit host immune processes but also recruit human neutrophils to the site of infection. This new insight allows us to better understand complex interactions between host and *S. aureus* pathological processes.

We are also interested in better understanding other commensal bacteria, especially the biggest commensal community in the gut. There are trillions of microbial organisms colonized in the human gut that form the gut microbiota. A large amount of studies have shown that the gut microbiota play an essential role in maintaining homeostasis and have a profound effect on the development and maturation of the immune system. **In chapter 4**, using the same screening method as **chapter 3**, we identified and characterized a secreted multi-domain protein of *Ruminococcus*, which we termed ProteinR, act as an new immunoglobulin binder. ProteinR binds the Fab-domain of human antibodies (IgA, IgM and IgG) with high affinity and efficiently blocks Fc mediated phagocytosis *in vitro*. Furthermore, ProteinR forms complexes with immunoglobulins that induced neutrophil ROS production. In addition, the intriguing observation that ProteinR like genes were found in different commensal bacterial species suggests that ProteinR may play a powerful role not only for antibody purification, but also may contribute to balance the colonization of commensal gut microbiome.

"*Everything is a double-edged sword*". Immune evasion mechanisms also can be targeted or exploited by future therapeutics. This is required in order to prevent or treat bacterial infections and inflammatory diseases. In **Chapter 5**, we use the immune complex-mediated Arthus reaction model that results in an inflammatory response and neutrophil recruitment that is mainly C5a mediated. We show that CHIPS can inhibit hC5aR1 mediated neutrophil migration in hC5aRKI mice in vivo. This indicates the therapeutic potential of CHIPS as a C5aR1 inhibitor in vivo. However, administration of CHIPS in human subjects led to immune complex formation with circulating anti-CHIPS antibodies and induced adverse effects that dampen the use of CHIPS in its current form as a therapeutic candidate. Nevertheless, future development of small homologue molecule anti-inflammatory drugs based on bacterial immune interaction molecules could very well be potential new candidates.

In **Chapter 6**, the results presented in this thesis are discussed in a broader context. In short, the research chapters discussed the role of newly identified immune interaction proteins and the role of diverse neutrophil receptors, as well as their therapeutic implications. This will pave the way for treating infections and inflammatory diseases.

# 7

# Nederlandse samenvatting

Het menselijke lichaam is een constant veranderend ecosysteem dat bestaat uit biljoen micro-organismen dat bekend is als het microbioom. Bij gezonde mensen bestaat er een symbiotische relatie met deze micro-organismen. Wanneer deze balans wordt verstoord zullen sommige symbiotische bacteriën transformeren in pathogene bacteriën die een infectie veroorzaken, lokaal en zelfs systemisch. Met name door overmatig gebruik van antibiotica zullen sommige, normaal ongevaarlijke, commensalen bacteriën, in toenemende mate virulent en resistent worden tegen antibiotica. Zulke bacteriën worden ook wel "super bug" genoemd,. Infecties veroorzaakt door multiresistente bacteriën vormen een belangrijk risico voor de volksgezondheid. Wanneer bacteriën eenmaal de gastheer binnendringen arriveren neutrofielen (ook wel bekend als PMN of polymorfkernige leukocyt), de meest voorkomende fagocyt in het menselijk bloed, als eerste bij de infectie haard en zorgen daar voor de primaire afweer tegen de binnengedrongen bacteriën. De neutrofielen spelen een cruciale rol in de afweer tegen bacteriële infecties en dragen bij aan het orkestreren van de ontsteking.

De primaire focus van dit proefschrift ligt bij het bestuderen van de interactie tussen neutrofiel en pathogeen, om deze kennis te kunnen toepassen in de ontwikkeling van therapieën voor infecties en ontstekingsreacties. In **hoofdstuk 1** beschrijven we allereerst de rol van de neutrofiel in de strijd tegen bacteriële infecties. Vervolgens beschrijven we het arsenaal van bacteriële "immuun ontsnapping" strategieën die bijdragen aan resistentie tegen een aanval door neutrofielen. Als derde benadrukken we toekomstige onderzoek perspectieven ter verbetering van onze kennis omtrent neutrofiel functie en bacteriële pathogenese.

Neutrofielen gebruiken een divers scala aan oppervlakte receptoren voor het herkennen van microbiële infecties en het in stand houden van de neutrofiel homeostase. Wanneer de neutrofiel te zwak reageert op een stimulus is de gastheer gevoeliger voor de infectie zoals bijvoorbeeld het geval is bij neutropene kanker patiënten die vaker last hebben van een *S. aureus* en *E. Coli* infectie. Daar tegenover, wanneer de neutrofiel te sterk reageert kan dit bij de gastheer schade veroorzaken. Dit treed onder andere op bij artritis, COPD en hart- en vaatziekten. Daarom zijn er nieuwe behandel mogelijkheden nodig on de neutrofiel in balans te brengen. We weten dat de neutrofiel wordt gereguleerd door verschillende groepen van receptoren waarvan sommigen de cel stimuleren en anderen de cel activiteit remmen. Het is aannemelijk dat de neutrofiel wel 100 verschillende receptoren aan het oppervlak bezit, waarvan een aantal goed gekarakteriseerd is terwijl basale kennis ontbreekt voor anderen. Dit betekent dat het volledige repertoire aan strategieën om de neutrofiel te moduleren nog onderbelicht is. Studies hebben





aangetoond dat sommige "leukocyte immunoglobulin-like receptors (LILRs)" de neutrofiel kunnen moduleren, maar degelijke kennis omtrent expressie en functie van alle LILRs op de neutrofiel ontbreekt. In **hoofdstuk 2** laten we zien dat LILRA2, LILRA6, LILRB3, en LILRB4 tot expressie komen aan het oppervlak van de neutrofiel terwijl LILRB2 tot expressie komt in de granula. Daarnaast laten we zien dat de LILR's in staat zijn de FcγR gemedieerde neutrofiel functies te moduleren, zoals productie van reactieve zuurstof metabolieten en fagocytose en doden van microben. We waren ook in staat om cel lijnen te genereren die LILRs tot expressie brengen aan hun oppervlak en cel lijnen die dat tevens koppelen aan een activatie reporter construct. Deze cel lijnen kunnen nuttig zijn in de toekomst om te achterhalen welke LILR(s) een belangrijke rol spelen tijdens een bacteriële infectie en om de biologische liganden voor de LILRs te identificeren.

*Staphylococcus aureus* (*S. aureus*) is een van de meest voorkomende veroorzaker van infecties in zowel het ziekenhuis als de gemeenschap. Om de gastheer beter te kunnen koloniseren en te overleven in die vijandige omgeving, zoals het menselijk lichaam, heeft *S. aureus* verschillende moleculen ontwikkeld die een interactie aangaan met het immuun systeem om daaraan te kunnen ontsnappen. In **hoofdstuk 3** hebben we een robuuste binding selectie strategie ontwikkeld met behulp van fagen die uitgescheiden eiwitten tot expressie brengen. Deze methode vormt daarmee een unieke strategie om nieuwe *S. aureus* eiwitten te ontdekken die een interactie aangaan met de neutrofiel. In tegenstelling tot vele andere beschreven immuun ontsnapping eiwitten die de immuun respons remmen, hebben we het stafylokokken superantigeen lijkend eiwit 13 (Staphylococcal superantigen-like protein 13; SSL13) gevonden met neutrofiel aantrekkende en activerende functie via de FPR2 receptor. SSL13 behoort tot een familie van 14 eiwitten die worden uitgescheiden en waarvan bekend is dat ze een functie hebben in ontwijken van het immuun systeem. Dus SSL13 is een uniek lid van die SSL familie dat niet behoort tot de klasse van immuun ontwijking, maar juist een pathogeen alarmerend molecuul is voor het immuun systeem. Daarmee geeft onze studie aan SSL13 een nieuw concept voor de SSL familie: de SSLs kunnen niet alleen het humane immuun systeem dempen maar ook neutrofielen rekruteren naar de plaats van ontsteking. Dit nieuwe aspect draagt bij aan een beter inzicht in de complexe interactie tussen gastheer en *S. aureus* pathologische processen.

Wij zijn ook geïnteresseerd in een beter begrip van andere commensalen bacteriën, en met name de grootste commensalen gemeenschap in onze darm. De humane darm wordt gekoloniseerd door biljoenen micro-organismen die tezamen de darm microbiota vormen. Vele studies hebben aangetoond dat de darm microbiota een essentiële rol speelt in het handhaven van een normale homeostase en een diepgaand effect hebben op de ontwikkeling en rijping van ons immuun

systeem. In **hoofdstuk 4** hebben we eenzelfde faag screenings methode gebruikt als beschreven in **hoofdstuk 3**. Hiermee hebben we een multi-domein eiwit van *Ruminococcus* geïdentificeerd en gekarakteriseerd als een nieuw immunoglobuline bindend eiwit dat wij ProteinR hebben genoemd. ProteinR bindt het Fab-domein van humane antistoffen (IgA, IgM, en IgG) met een hoge affiniteit en zorgt voor efficiënte blokkade van Fc gemediëerde fagocytose in vitro. Daarnaast vormt ProteinR een complex met immuunglobulinen dat resulteert in de productie van reactieve zuurstof metabolieten door neutrofielen. De intrigerende ontdekking dat ProteinR lijkende genen werden gevonden in verschillende commensalen bacterie species suggereert dat dit eiwit, naast een krachtige functie als immuunglobuline isolerend eiwit, ook zou kunnen  bijdragen aan het in stand houden van de balans tussen gastheer en microbiota in de darm.

"Alles is een dubbelzijdig zwaard".  Het is goed vast te houden aan het principe dat onderzoek bijdraagt aan praktische toepassingen. Het bestuderen van immuun ontwijkende strategieën kan in de toekomst leiden tot ontwikkeling van nieuwe therapeutische middelen die kunnen worden toegepast voor preventie of behandeling van infecties of ontstekingsreacties. In **hoofdstuk 5** hebben we een immuuncomplex gemediëerde Arthus reactie gebruikt als model voor een C5a afhankelijke ontstekingsreactie met rekrutering van neutrofielen. We laten zien dat CHIPS in humane C5aR Knock-In muizen de hC5aR1 gemediëerde migratie van neutrofielen in-vivo kan remmen. Echter, het toedienen van CHIPS aan mensen resulteerde in de vorming van immuuncomplexen met de aanwezige circulerende anti-CHIPS antilichamen. Dit veroorzaakte ongewenste bijwerkingen die het gebruik van CHIPS in zijn huidige vorm als therapeutische kandidaat ondermijnen. Desalniettemin, de ontwikkeling van kleine afgeleide homologe moleculen gebaseerd op bacteriële eiwitten met immuun modulerende eigenschappen zouden in de toekomst potentiele nieuwe onstekingsremmers kunnen vormen.

Tenslotte, bediscussiëren we in **hoofdstuk 6** de gevonden resultaten in een bredere context. Samengevat geven de verschillende hoofdstukken inzicht in de rol van de nieuw geïdentificeerde eiwitten die een interactie aangaan met het immuun systeem, de rol daarin van verschillende neutrofiel receptoren en de mogelijke therapeutische implicaties. Uiteindelijk kan dit bijdragen aan behandeling van infecties en ontstekingsziekten.

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**纪元**，一路从吉大相遇相知到荷兰，过去所有的点滴会相伴在我们最美好的回忆里。**宏凯**，谢谢你在过去几年的所有帮助和鼓舞以及适时的打击，很开心你和纪元终修成正果，也有了可爱的米勒，愿你们一家人永远快乐！咱们北京未完待续。

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# Curriculum Vitae

Yuxi Zhao was born on July 5th, 1988, in Henan, China. In 2007 she completed her high school study at Beitun public high school, 10th Division of Agriculture, Beitun, Xinjiang, China. In the same year, she was admitted to study Veterinary Medicine in Tarium University, Xinjiang, China. In 2011, she received her bachelor degree with the honor of outstanding graduate. After this, she was admitted to the Institute of Zoonosis at Jilin University, which is the Key Laboratory for Zoonosis Research, Ministry of Education, China, to obtain her Master degree. The lab where she performed her Masters is led by Prof. Qisheng Peng who focuses on understanding the regulation of the inflammatory response of macrophages in *Brucella* infection. Since then, her interest grew for bacterial infection and immunity. In 2014, she got a four year grant from the Chinese Scholarship Council (CSC) for a PhD study in Prof. Jos A. G. van Strijp's group, at the UMC Utrecht, the Netherlands. Her research focused on exploring the interactions between neutrophils and bacteria, which resulted in this thesis. Yuxi will switch her scientific career from innate immunology to adaptive immunology as a postdoctoral fellow in the lab of Prof. Dong at Tsinghua University in Beijing, China.





# List of publications

## Related to this thesis

Zhao, Y., van Kessel, K. P., de Haas, C. J., Rogers, M. R., van Strijp, J. A., & Haas, P. J. A. (2018). Staphylococcal superantigen-like protein 13 activates neutrophils via formyl peptide receptor 2. Cellular microbiology, 20(11), e12941.

## Other publications

Zhao, Y., Cui, G., Zhang, N., Sun, W., & Peng, Q. (2012). Lipopolysaccharide induces endothelial cell apoptosis via activation of Na+/H+ exchanger 1 and calpain-dependent degradation of Bcl-2. Biochemical and biophysical research communications, 427(1), 125-132.

Cui, G., Zhao, Y., Zhang, N., Liu, Z, Sun, W., & Peng, Q. (2013). Amiloride attenuates lipopolysaccharide-accelerated atherosclerosis via inhibition of NHE1-dependent endothelial cell apoptosis. Acta Pharmacologica Sinica, 34(2), 231.