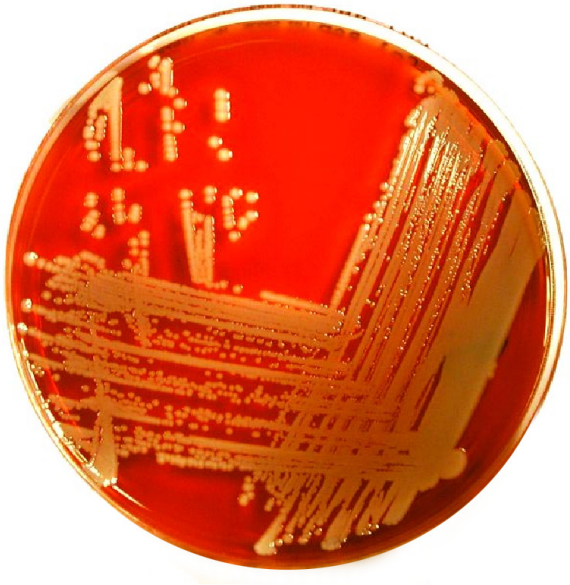
Immune evasion in *Staphylococcus aureus*



Olivier Gerrit de Jong
Studentnr. 0215430

Supervisors: J. Bestebroer & C. J. C. de Haas

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Introduction

Staphylococcus aureus is mentioned in the media more often than most other bacteria. This is mainly because *S. aureus* is one of our most dreaded adversaries in the battle against bacterial infections since the discovery of the first antibiotic, penicillin. Every time a new antibiotic was discovered, *S. aureus* became resistant to it. Nowadays there are only 2 antibiotics that can be used to fight an *S. aureus* outbreak within a hospital and *S. aureus* strains that are resistant to these antibiotics have already started to emerge. *S. aureus* is also known for a live-threatening syndrome that threatens tens of thousands of lives a year: Toxic Shock Syndrome, a syndrome often caused by tampons that are too absorbent allowing *S. aureus* to grow and release toxin. And not only has *S. aureus* been in the media because of its infections in hospitals, it is also one of the most common causatives of food poisoning. All in all, *S. aureus* is quite a hazardous pathogen.

Interestingly enough, *S. aureus* is not a contagious disease that is spread by coughing or coming in to contact with diseased people. *S. aureus* is a skin commencal that is persistently carried by a large part of the population. This raises many questions: why do some carriers not develop disease while others do, how does *S. aureus* develop resistance against antibiotic treatment so rapidly, and most importantly: if *S. aureus* has the potential of becoming such a dangerous pathogen, how is it able to persistently colonize so many of us?

To answer the last question, it is important to understand the responses of the human immune system to bacterial infections in general. Like all higher mammals, the human immune system has innate and adaptive immune responses. While the adaptive immune response allows us to mount strong and specific responses against recurring infections of pathogens, the innate immune response allows us to mount a less specific response which plays a larger role in fending of acute infections. In this acute response neutrophils and the complement system play a very important role. It is therefore not surprising that through the course of evolution *S. aureus* developed multiple ways to elude both of these major players in this immune response. This thesis will therefore first focus on describing the immunological reactions of the complement system and neutrophils involved in controlling a staphylococcal infection. Subsequently, different proteins and strategies used to elude the complement system and neutrophils will be discussed by studying primary data.

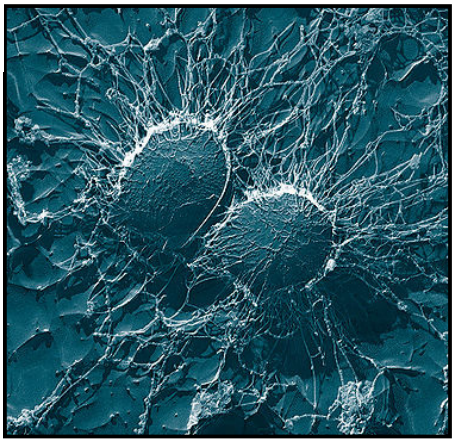
Chapter 1 *Staphylococcus aureus* and the human immune defenses

1.1 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a member of the genus *Staphylococcus* within the family of *Micrococcaceae*. This Gram-positive bacterium with a spherical shape has a size of 0.2-2 µm in diameter and 2-8 µm in length (Fig 1A). *S. aureus* was first discovered in 1880 by Sir Alexander Ogston in Aberdeen, Scotland and was found in pus from surgical abscesses. (1) When plated out on a blood sheep agar plate *S. aureus* forms yellow or golden colonies, hence the name *S. aureus* (aureus is latin for golden). (2) In approximately 20% of the human population, the anterior nares are permanently colonized by *S. aureus*, it is estimated that it is transiently associated with 60% of the population. Under normal circumstances, the presence of this bacterium does not cause any harmful effects. In immunocompromised patients however, infection with *S. aureus* can lead to severe symptoms and even death. Therefore, *S. aureus* is considered an opportunist as well as a pathogen. (3)

The genome of *S. aureus* consists of a circular genome of approximately 2.8 Mb, which has a low G+C content of only 33%. It contains 2600 open reading frames, covering 84.5% of the genome. Many virulence factors appear to be associated with mobile elements, either through plasmids, transposons, prophages or pathogenicity islands. (4) Peptidoglycan takes up 50% of the weight of the cell wall. Covering this peptidoglycan layer is a large capsule of polysaccharides. There are several surface proteins that play an important role in host colonization through the binding of extracellular matrix molecules. These proteins are anchored in the cell wall, span the peptidoglycan layer and the capsule and their binding domains protrude from the surface of the capsule. (2) *S. aureus* is also known to secrete proteins, which are usually toxins that play an important role in pathogenicity (Fig 1b). (3)

A



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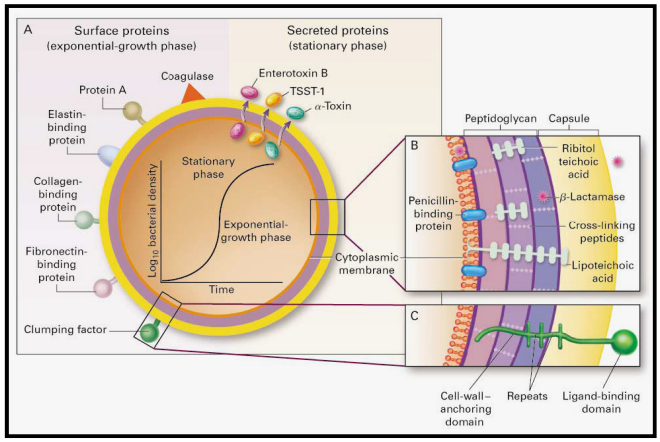

FIGURE 1. *Staphylococcus aureus structure*. **A)** Electron microscopy picture of *S. aureus*, at a magnification of 50.000 times. (5) **B)** Schematic representation of *S. aureus*. (2).

1.2 Clinical symptoms

Under normal conditions, carrying *S. aureus* does not lead to any symptoms. Problems generally occur only in immunocompromised patients. Naturally persistent carriers are at higher risk of infection when immunocompromised, as they are constantly exposed to *S. aureus*. (6) In other cases, patients come in contact with *S. aureus* in hospitals via other patients or via staff. Infections can then occur when the skin or mucosal tissues are damaged, allowing *S. aureus* to enter the tissue or the bloodstream. (2)

Once bacteremia (the presence of bacteria in blood) has occurred, *S. aureus* is able to spread to other tissues. Since *S. aureus* carries surface proteins that bind to extracellular matrix proteins, it is believed that tissue can be entered from the bloodstream through binding platelet-fibrin thrombi at sites were endovascular damage occurred. (7) It has been proposed that surface proteins that act as adhesins also allow *S. aureus* to bind to epithelial cells and then, through endocytosis, invade epithelial cells and access the underlying tissues. (8). Once the underlying tissue has been invaded, abscesses are formed, which induce inflammatory responses. These abscesses are also referred to as metastatic foci. *S. aureus* is also known to spread to joints, bones, kidneys and lungs. (7) In approximately 1 out of 3 cases of *S. aureus* infections, endocarditis (inflammation of the inner layer

of the heart) occurs. Since the heart valves do not receive a separate blood supply, they cannot directly be reached by neutrophils. The mortality rate for endocarditis, if caused by *S. aureus*, is over 50%. (2)

During an *S. aureus* infection, the most drastic symptoms are caused by the secretion of pyrogenic-toxin superantigens. These superantigens bind directly to invariant regions of major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and the T cell receptor (TCR) on T cells. As a result an expansion of clonal T cells occurs. This leads to a substantial release of cytokines by both cell types that causes tissue damage and can eventually lead to toxic shock syndrome. As a result of the cytokine storm induced by these superantigens, patients experience high fever and low blood pressure. This typically leads to confusion and headaches and can, and often will, rapidly progress to capillary leak, coma, multi-organ failure and eventually death. (2)

1.3 Human defenses: The complement system

To fend off infections, the human has an elaborate system of defences called the immune system. The human immune system consists of the adaptive immune system and the innate immune system. The adaptive immune system is able to generate specific responses against pathogens and build up an immunological memory so that the invading pathogen can quickly be eliminated if it would infect the body a second time. The non-specific innate immune response plays a very important in containing and killing pathogens in the earlier stages of infection. (3, 9) During an acute response to an infection the innate immune system deploys humoral and cellular components to mark and kill the invading pathogen. The innate system has several types of cells to kill certain types of pathogens, as well as to activate the adaptive immune system. In *S. aureus* infections, neutrophils play a major role. The humoral component of the human innate immune system is also known as the complement system and plays a central role in our innate immune system. It recognizes foreign cells, is involved in the removal of cellular debris and plays an important role in activating the adaptive immune system. The complement system is based on a well-balanced network of proteins that interact with each other. This cascade is usually initiated upon an encounter with "non-self" structures. The complement system can be activated via three separate pathways: the classical pathway, the lectin pathway and the alternative pathway. *S. aureus* has been

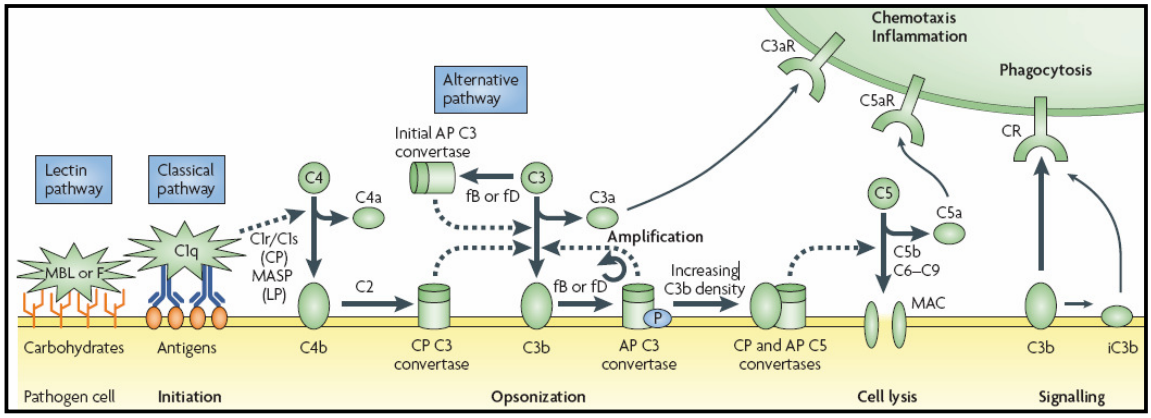

FIGURE 2. Schematic representation of the complement system. The lectin, classical, and the alternative pathway lead to the assembly of C3-convertases. The C3 convertases are then amplified, chemoattractants C3a and C5a are released and the Membrane Attack Complex can be formed on the surface of the pathogen. (10)

shown to activate all 3 pathways of the complement system. (10)

The classical pathway is initiated by the recognition of so-called antigen-antibody complexes. These antigen-antibody complexes are immunoglobulins (Igs) made by the adaptive immune system. IgM and IgG can trigger this pathway by binding to a specific structure on a pathogen. They trigger binding of C1q, a hexameric component of the complement system, which leads to the activation of 2 C1r molecules. These C1r molecules then both cleave C1s, thus creating a C1r2s2 complex. This complex cleaves C2 and C4 into C2a and C2b, and C4a and C4b. C2b and C4b bind and form a C4b2b complex, which is also known as the C3-convertase. (10, 11)

The lectin pathway is quite similar to the classical pathway, but it is initiated when carbohydrate ligands on the surface of a pathogen are recognized by mannose-binding lectin (MBL) and ficolins. Upon binding the MBL-associated serine proteases 1 and 2 (MASP-1 and MASP-2), which are highly similar to C1r and C1s, respectively, cleave C2 and C4 to form the C3 convertase made of C4b2b. (10, 11)

The alternative pathway is based on spontaneous hydrolysis of C3 into C3a and C3b. If, by chance, a pathogen surface is close enough, C3b will covalently bind to its membrane. Hydrolysis of C3 is significantly increased by

the presence of hydroxyl-rich pathogen surfaces. In the presence of factor D surface-bound C3b can bind and cleave factor B. The generated C3bBb complex is also a C3 convertase. (10, 11)
Thus, all three pathways result in the generation of C3 convertases. These convertases cleave C3, which results in the release of the chemoattractant C3a and the deposition of C3b on the microbial surface, C3b is able to bind to the pathogen's surface and plays an important role in opsonization: neutrophils/macrophages recognize C3b using their Complement Receptor 1 (CR1), inducing phagocytosis of the pathogen. As was previously described, C3b can also activate the alternative complement pathway, which then serves as an amplification loop in the complement cascade. The C3 convertase can bind an additional C3b molecule to form the C5 convertase. The C5 convertase cleaves C5 into C5a and C5b. C5a functions as an anaphylatoxin, which elicit pro-inflammatory responses and chemotaxis; it induces the release of histamine from mast cells, attract neutrophils and increases local vascular permeability, leading to a local accumulation of neutrophils. C5b binds to the pathogen surface and recruits C6 – C9, thereby creating the Membrane Attack Complex (MAC). The MAC is responsible for direct killing of Gram-negative bacteria by formation of pores in their membrane, resulting in cell lysis. However, Gram-positive bacteria such as *S. aureus* are rather resistant to this response due to their thick peptidoglycan cell wall. Therefore, the generation of C3a and C5a is more important in *S. aureus* infections. (10, 11)

If this cascade would occur on harmless host tissue, this can have severe effects on the host body. Therefore, several protein are involved in regulatory feedback pathways. These proteins are called regulators of component activation (RCAs) or complement control proteins (CCP). A very effective method of blocking the complement cascade is by inactivating C3b when it has bound to the surface of a host cell. This is performed by factor I, which degrades C3b into iC3b. Cofactors for this process are factor H, which is present in host cell membranes, MCP and DAF. iC3b is no longer able to bind with factor B, preventing further amplification of the complement cascade. iC3b does however still retain its ability to opsonize surfaces. In presence of CR-1, iC3b can be degraded even further into C3d, which is proposed to play a role in B-cell activation, shifting the immune response from innate to adaptive. Factor I has also been described to degrade C4b, preventing the assembly of C3 convertases generated by the classical and lectin pathway as well. Another protein that inhibits C4b is C4 binding protein (C4BP), which cleaves the C4 convertase, effectively preventing further production of C4b. Like C4BP, C1-inhibitor also inhibits the classical- and lectin pathway. C1-inhibitor deactivates C1r and C1s, MASP-1 and MASP-2, blocking both pathways in a very early stage. This protein is not bound to host cell surfaces but is present in low concentrations in a soluble form. Finally, CD59, S-protein and clusterin prevent the formation of the MAC, preventing the formation of pores in host cells when C5b is only present in very low concentrations (for example due to incidental hydrolysis of C5). (10, 9)

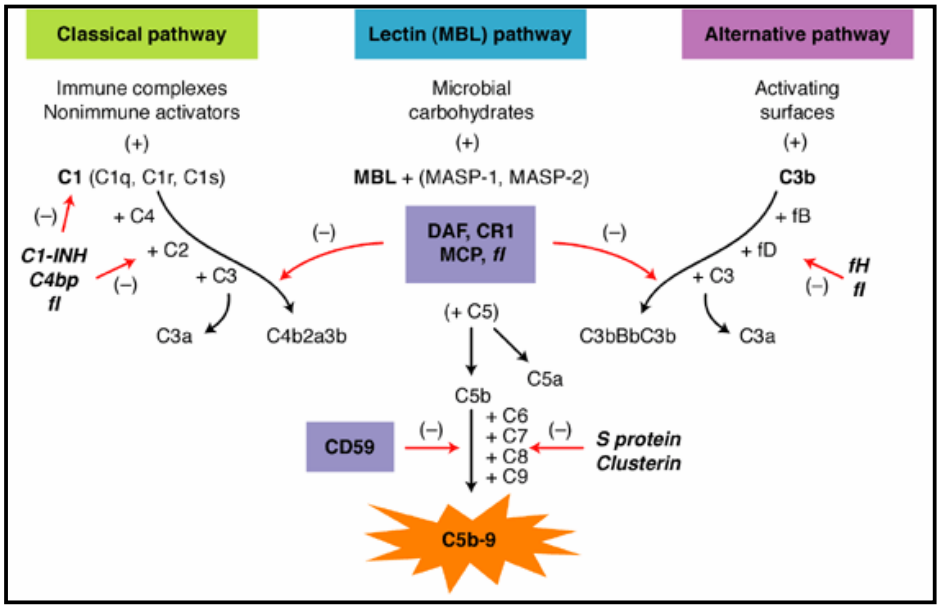

FIGURE 3. Activation and regulation of the complement system. A schematic overview of the classical-, lectin- and alternative pathway and their regulators. (9)

Regulators of complement activation are finely tuned in to one another and any defects within this regulation often leads to specific diseases and syndromes. These proteins can either be present in soluble form or in membrane-bound form and prevent or actively inhibit the activation of the complement cascade as a response to host tissue. These proteins are often species-specific, preventing pathogens to be able to develop proteins that universally inhibit host complement systems. (10, 11)

1.4 Human defenses: Neutrophils

The complement cascade by itself is often not sufficient to clear out an infection. Therefore the complement system also opsonizes pathogens and induces chemotaxis: C3a and C5a, anaphylatoxins, are produced at the site of infection, resulting in recruitment of large numbers of leukocytes. The leukocytes that migrate towards the source of infection are predominantly neutrophils. Neutrophils kill the invading pathogens by phagocytosis. (12)

Under normal conditions, neutrophils circulate in the bloodstream. When they pass through capillaries near a site of inflammation however, they migrate to the place of infection via a process called extravasation. (13) The first step in this process is called rolling an tethering; the slow blood flow through these capillaries allows a loose adhesion, also called tethering. This causes the neutrophils to "roll" alongside the endothelium. This rolling is mediated by interactions between selectins and their specific sialyl carbohydrates ligands. (12) Upon stimulation by underlying inflamed tissues, the endothelium rapidly translocates stored P-selectin to its surface, allowing neutrophil P-selectin glycoprotein ligand-1 (PSGL-1) to bind. This step is vital for neutrophil rolling, which improves the chance of neutrophils to become activated by chemokines such as interleukin-8 (IL-8) as they are presented by the capillary endothelial cells. Upon encountering chemokines, rapid integrin activation is induced in the neutrophils. The integrins lymphocyte function-associated antigen 1 (LFA-1), Integrin alpha M (Mac-1) and integrin alpha 4 beta 1 (VLA-4) can bind to their endothelial counterparts: intercellular adhesion molecule 1 (ICAM-1), intercellular adhesion molecule 2 (ICAM-2) and vascular cell adhesion molecule-1 (VCAM-1), respectively. Upon binding, firm adhesion of the neutrophils to the endothelial cells is observed. The neutrophils will then transmigrate through the extracellular matrix between the endothelial cells. This is facilitated by proteolytical processes and is LFA-1 – ICAM-1 mediated. (12) Further migration during transendothelial migration and underlying tissue is guided by a gradient of chemoattractants such as formylated peptides released by bacteria, interferon gamma (IFN-γ) or IL-8 released by the inflamed tissue, or C5a produced during complement activation. Migration along the increasing gradient of chemoattractants, which is called chemotaxis, leads the neutrophils to the site of inflammation, where they can fend off the infection through phagocytosis of the pathogen. (12, 13, 14)

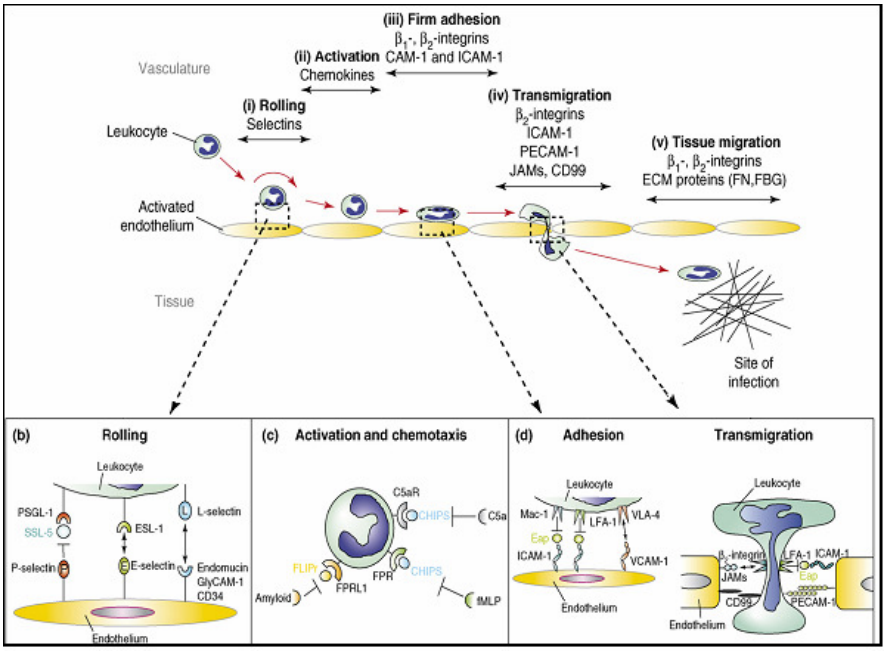

FIGURE 4. Schematic representation of neutrophil extravasation. **A)** Overview, displaying the order of events: rolling, activation, adhesion, transmigration and chemotaxis. **B)** Binding that mediates rolling: selectins play a key role. **C)** Important chemoattractans responsible for activation and chemotaxis: formylated Met-Leu-Phe (fMLP), C5a and FPRL1 ligand. **D)** Adhesion (ICAMs and VCAM) and modes of transmigration. (14)

For phagocytosis to occur, neutrophils must first recognize the pathogen. This can take place by recognition of IgG-coated targets by Fcγ-receptor FcγIIA. When IgG is abundant, FcγIIA accumulates, leading to phosphorylation of their immunoreceptor thyrosine-based activation motifs. This leads to a signaling cascade which activates Rho proteins, leading to membrane protrusions around the surface marked with IgG forming a phagocytic cup, and PI3-kinase activation, leading to the fusion of the membranes. This causes the phagocytic cup to close and engulf the pathogen. This process also takes place after recognition of iC3b by C3-R. When C3-R abundantly binds to iC3b, the opsonized pathogen "sinks" into the cell. Since C3-R mediated uptake alone does not initiate the oxidative burst, it has been proposed that both mentioned mechanisms operate synergistically. Observation of a decrease in IgG-mediated uptake in C3-R deficiencies further support this theory. (13, 14)

Neutrophils also recognize pathogens using their Toll Like Receptors (TLRs). Neutrophils express TLR-2, allowing them to recognize lipoteichoic acid (LTA) and peptidoglycan. In fact, it has been shown that introducing peptidoglycan from *S. aureus* causes neutrophils to change in adhesion, alter the expression of surface proteins and induces an oxidative burst.

After phagocytosis, neutrophils kill bacteria using an array of factors which can be classified in oxygen-dependent and oxygen-independent mechanisms. (15) Oxygen-independent agents consist of proteases, antimicrobial proteins and enzymes. Proteases like neutrophil elastase and cathepsin G degrade bacterial proteins including virulence factors, decreasing the pathogenicity of the bacterium. Antimicrobial proteins and peptides are defensins which disrupt membrane integrity by binding to the bacterial membrane and forming pores, permeability increasing protein, and the lysozyme which damages the peptidoglycan layer by catalyzing hydrolysis of its components. These components are stored by neutrophils in granules, allowing neutrophils to expose pathogens to high concentrations of these agents. The process of releasing these agents from their granules is called degranulation. (12, 16)

The oxygen-dependent mechanism relies on the exposure of an oxidative burst to the pathogen. After phagocytosis the components of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex assemble at the membrane of the phagosome. The NADPH oxidase complex starts producing O_2^-, which is converted into hydrogen peroxide by superoxide dismutase. The hydrogen peroxide is subsequently converted into hyperchlorous acid. While the reactive oxygen species effectively degrade the bacterial membrane through an oxidative chain reaction, hyperchlorous acid reacts with cholesterol, fatty acid groups, proteins, DNA and RNA. The combination of the exposure to these oxygen-dependent and oxygen-independent mechanisms lead to the degradation of the bacterium (Fig. 5). (16)

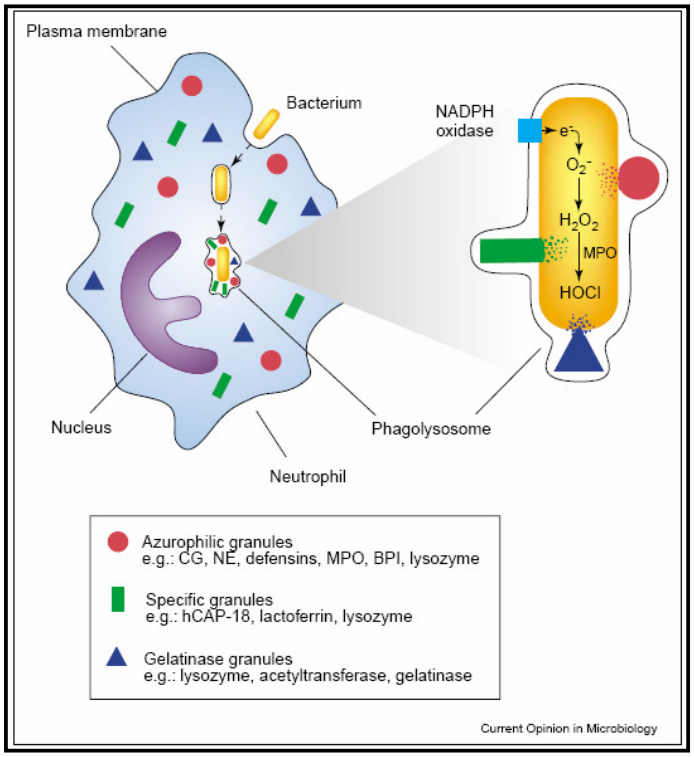

FIGURE 5. Granules within neutrophils. The 3 types of granules contain specific compounds which are, in combination with NADPH oxidase, utilized to digest bacteria within the phagolysosome. (16)

Chapter 2 Complement evasion

The complement system is involved in chemotaxis, opsonization and disrupting membrane integrity of pathogens. It is often the initial step taken by the immune system to fend off a pathogen. Therefore, it is not surprising that a large amount of agents that *S. aureus* utilizes to evade the host immune system are aimed at components of the complement system. In this chapter these proteins will be discussed.

2.1 Extracellular fibrinogen-binding protein (Efb)

Efb was the first C3b-binding protein that was discovered in *S. aureus*. (14) Since *S. aureus* causes persistent infections, it was hypothesized by Lee at al that it would counter complement activity. It was already shown that *S. aureus* activated all three pathways of the complement cascade, so if *S. aureus* had a protein that disabled complement activity it would probably target a specific part of the complement system involved in all three pathways. (10, 11) As can be seen in Fig. 3, all 3 pathways merge after their specific C3 convertases convert C3 to C3a and C3b. (10) Thus, the first agent that is involved in all three pathways in the complement cascade is C3b. Bacteria and supernatant of *S. aureus* were fractionated using SDS-PAGE and probed with labeled C3. The results were compared to fractionated bacteria *S. carnosus* and *E. coli*, both bacteria that are unable to persistently infect humans (Fig. 6A). Only in the fractionated supernatant and bacteria of *S. aureus* a 19 kDa protein was found that would bind C3. Analysis showed that this protein had earlier been described as a fibrinogen-binding protein, called Efb. Further studies showed that Efb inhibited lysis of red blood cells induced by both the classical- and the alternate complement pathways (Fig. 6B). (17)

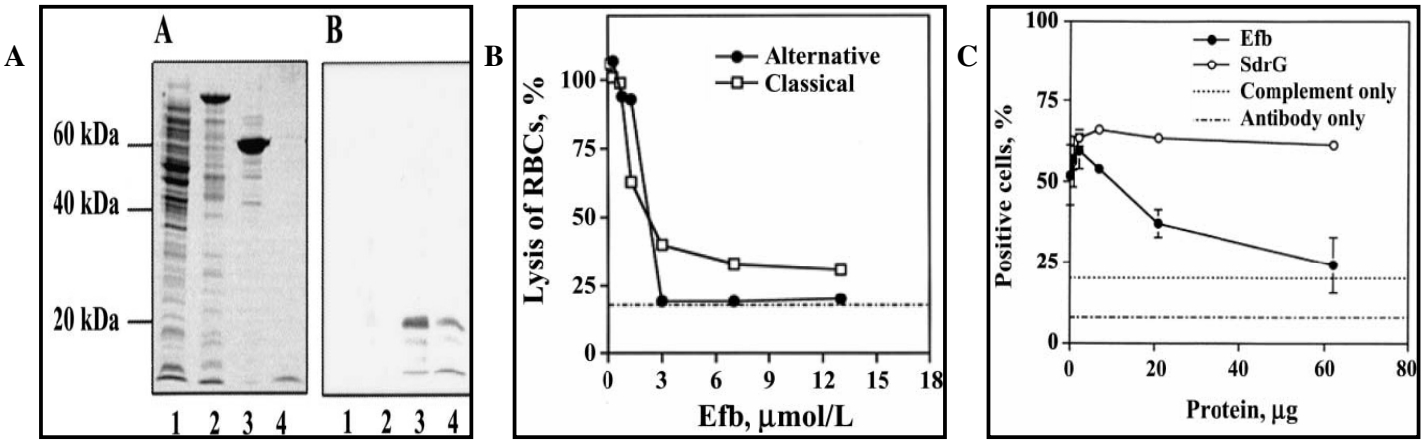

FIGURE 6. Extracellular fibrinogen-binding protein binds to C3b and inhibits lysis by the complement cascade and complement-mediated phagocytosis **A)** Detection of a *Staphylococcus aureus* C3-binding protein. A: SDS page stained with Coomassie blue. B:Polyvinylidene fluoride membrane, probed with labeled C3. Lane 1: *E. coli*, lane 2: *S. carnosus*, lane 3: *S. aureus* and lane 4: *S. aureus* supernatant. In lane 3b and 4b a 19-kD band can be seen that binds to C3. Smaller bands are assumed to be degradation products of the 19 kDa band (17) **B)** Dose-dependent effect of Efb on complement-mediated phagocytosis. This experiment is too elaborate to briefly explain here. For methods, see (17). The presence of Efb seems to have a dose-dependent effect on induced phagocytosis by both the alternative and the classical pathways. (17). **C)** The effect of Efb on complement-mediated opsonophagocytosis. Fluorescent Antigen-specific labeled beads were introduced to neutrophils for 30 minutes. Phagocytosis was then determined by measuring neutrophil fluorescence. The presence of Efb causes a significant decrease in phagocytosis. (17)

Since the complement cascade also leads to the accumulation of iC3b on the pathogen surface, it was studied whether the presence of Efb also inhibited complement-mediated phagocytosis (10). Fluorescent beads were opsonized with antigen-specific antibody and complement and introduced to terminally differentiated granulocytes (Fig. 6C). At the highest concentration of Efb measured, a decrease from 52% to 24% in phagocytosis was demonstrated. Further studies showed that Efb does not only bind to C3b, but to uncleaved C3 as well. (17) Since Efb is expressed constitutively, its function is most likely the countering of complement activation in the early stages of infection. Unsurprisingly, Efb is highly conserved among *S. aureus* strains. (18) Since Efb is also able to bind to fibrinogen it has been proposed that Efb might also serve as an adhesion protein next to having an immunomodulatory function. (18)
Thus, besides binding to fibrinogen, Efb also binds to C3 and C3b, thereby inhibiting opsonization and lysis by all three pathways of the complement cascade.

2.2 Clumping factor A (ClfA)

Like Efb, ClfA contained a significant similarity to several fibronectin-binding proteins of *S. aureus.* Since fibrin is one of the proteins that clots to implanted biomaterials at very high effectivity, this made ClfA a very interesting protein to study. (19) Many infections with *S. aureus* in hospitals are believed to occur due to "device-related infections" and this protein could perhaps explain this tendency. (20) Studies by Hair et al showed that the binding of *S. aureus* to fibrinogen-coated surfaces could dose-dependently be inhibited using immunoglobulins directed against the ClfA fibrin binding site, confirming that ClfA was responsible for the binding of *S. aureus* to fibrin. (20) The following 8 years little progress was made in the studying of ClfA, since most scientists believed that its complete function had been uncovered and fully described (21).

With the ever increasing occurrence of multi-resistant *S. aureus*, genotyping became increasingly important in giving valuable information to hospitals about the origin of the contamination as well as finding more specific and effective treatments against *S. aureus* infections. These studies also indicated that ClfA was an important virulence factor in specific clinical symptoms in *S. aureus* infections. When Palmqvist et al discovered that ClfA deficiency in *S. aureus* caused a decrease of symptoms in mice during infection, ClfA became a topic of interest once again. (22) When the effect of ClfA expression on phagocytosis by mouse macrophages was studied, a 2 – 3 fold increase in phagocytosis was shown in a ClfA deficient strain. (Fig. 7A) (22) Since ClfA has been shown to cause forming of bacterial clumps as a result of cross-binding fibrin, it was hypothesized that it would be more difficult for a macrophage to phagocytize large clumps of *S. aureus*. Clumping assays were performed and, as expected, no clumping was shown under any circumstances in the CflA deficient strain. (Fig. 7B) The wildtype strain, as expected, required the presence of fibrinogen to perform clumping. However, since phagocytosis occurred more in 10% mouse plasma then it did in 10% mouse serum (Fig. 7C) and clumping did not differ significantly, a decrease in phagocytosis as a result of clumping seemed very unlikely. On top of that, the highest difference in phagocytosis between both strains was observed under conditions were the wildtype *S. aureus* strain did not demonstrate any clumping at all. Thus it was concluded that ClfA inhibits phagocytosis of *S. aureus* by neutrophils, but not by clumping of bacteria. (22)

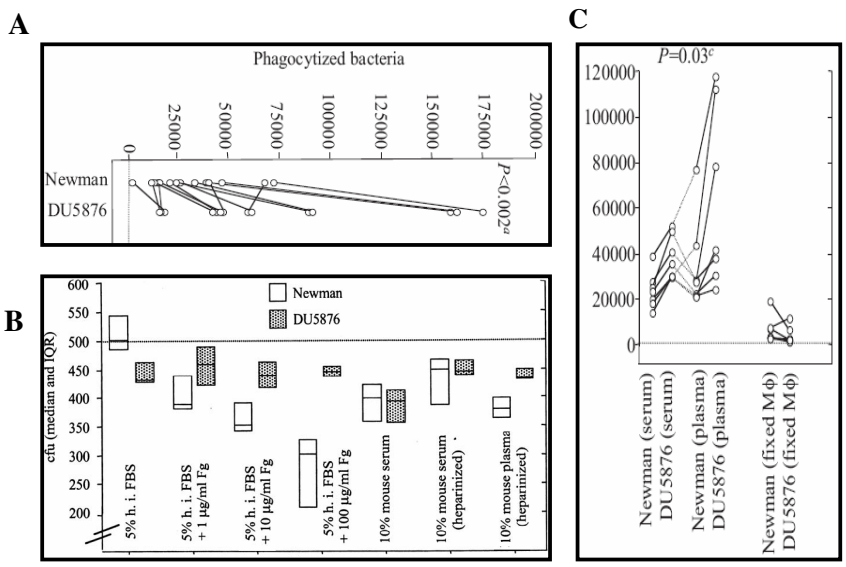

FIGURE 7. Clumping factor A inhibits phagocytosis by macrophages and induces clumping in the presence of fibrinogen. **A)** Phagocytosis of ClfA expressing (Newman) and ClfA-deficient (DU5876) *S. aureus* strains. Macrophages were incubated with *S. aureus* strains for 50 minutes at 37° C in the presence of 5% heat-inactivated fetal bovine serum. Afterwards, all extracellular *S. aureus* bacteria were killed using gentamicin-containing medium. Macrophages were then lysed and the lysate was plated out to determine the amount of *S. aureus* bacteria that were ingested. The deficiency of ClfA greatly induces *S. aureus* phagocytosis. (22) **B)** ClfA clumping assay using ClfA expressing (Newman) and ClfA-deficient (DU5876) *S. aureus* strains. The expected number of colony forming units (CFU) is indicated by the dashed line. The further the values deviate from this line, the more clumping has occurred. Clumping of the ClfA-deficient strain is mainly unaffected by the different circumstances. ClfA expressing *S. aureus* shows a dose-dependent increase of clumping in the presence of fibrinogen (Fg). There is no significant difference between presence of mouse serum and plasma serum. (22) **C)** Phagocytosis of ClfA expressing (Newman) and ClfA-deficient (DU5876) *S. aureus* strains in 8 – 10 % mouse serum or plasma. Experiment was performed in a similar manner as was performed in Fig. 7A. Fixed macrophages were added as a control. There is a significant increase in phagocytosis of *S. aureus* by macrophages in plasma when compared to serum. (22)

Quite recently, in a study were the supernatant of *S. aureus* was scanned for proteins that interacted with factor I, Hair et al isolated and identified a 50-kD fragment of the active A region from ClfA. (20) Since the cleavage of C3b into iC3b is mediated by factor I it was studied whether factor I was actually bound to the surface of *S. aureus*. (23) Using ELISA it was shown that *S. aureus* was indeed able to bind factor I to its surface when incubated in serum (Fig. 8A). Further studies showed that the only protein that was able to bind factor I was a 130-kD protein, which was identified as ClfA. Subsequently, using a recombinant form ClfA, rClfA, it was shown that C3b was cleaved into its inactive form iC3b by rClfA in the presence of factor I. (Fig. 8B). Consequently, the same effect could be demonstrated when *S. aureus* supernatant was used. After analysing the supernatant it was found that this was caused by a 55-kD protein which was found to be a fragment of ClfA, containing a part of the ClfA active region; region A. This fragment that is "shed" by *S. aureus* was named sClfA. When sClfA was incubated with purified C3b and factor I, iC3b production increased 9-fold (Fig. 8C). (20) Thus, next to being able to bind fibrin, ClfA is able to employ factor I to inactivate C3b as a membrane protein on the surface of *S. aureus* as well as in its immediate surroundings as a soluble form. This inhibits the complement cascade, decreasing the formation of the MAC, opsonization by iC3b and chemotaxis as a result of C3a and C5a production. (12)

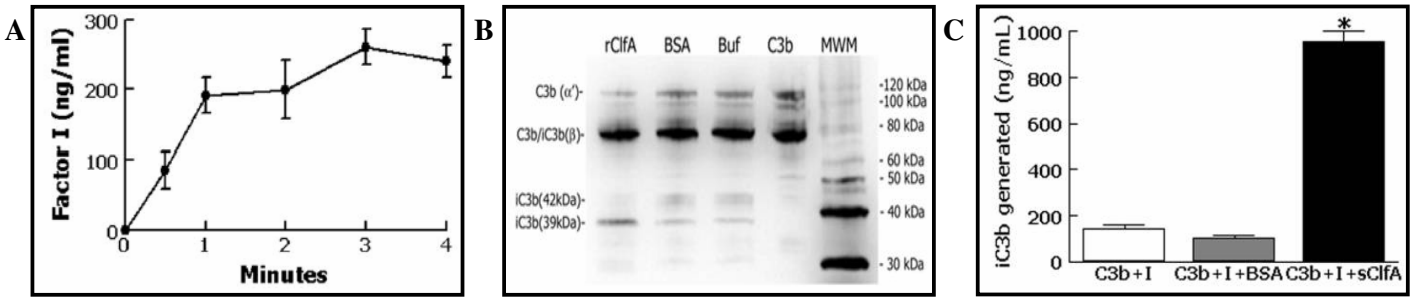

FIGURE 8. *S. aureus* binds factor I and Clumping factor A cleaves C3b into iC3b in the presence of factor I. **A)** Serum factor I binding to *S. aureus* in 10% human serum measured over time. Surface bound factor I was determined using ELISA. The primary antibody was a goat anti-human factor I antibody. Especially in the first minute a rapid increase in bound factor I is demonstrated. (23). **B)** Results of microtiter plates coated with recombinant ClfA which was incubated with purified C3b and purified factor I. rClfA shows a decrease in C3b (104-kD) and an increase in iC3b (39-kD) when compared to controls (BSA and buffer). This demonstrates that in the presence of factor I and rClfA, C3b is cleaved into iC3b. (23) **C)** sClfA incubated with purified C3b and purified factor I. A significant increase in generated iC3b is seen when sClfA is incubated with C3b and factor I when compared to C3b and factor I or C3b, factor I and BSA incubated under the same circumstances. This demonstrates that in the presence of factor I and sClfA, C3b is cleaved. (23)

2.3 Staphylokinase (SAK)

Like in Efb and ClfA, it was believed at first that the sole role of SAK was to interact with the extracellular matrix to aid in colonization and tissue migration. SAK is a 136 amino-acid extracellular protein that is located on pathogenicity island 5 in the *S. aureus* genome, and is assigned to a group of bacterial plasminogen activators (PA) that form complexes with plasminogen (PLG) and plasmin (PL). (24) These proteins do not interact with PLG and PL on an enzymatic level; they bind to it on a 1:1 ratio. These complexes then convert PLG into PL, its active form. PL, a serine protease, has been described to degrade components of the extracellular matrix as well as fibrin clots. Since SAK is an extracellular protein, surface-bound PLG can become active, causing a local production of PL. (25) At first it was suggested that the function of SAK was to induce local degradation of the extracellular matrix, allowing *S. aureus* to migrate through tissue. After it was illustrated that PL is also able to cleave IgG through hydrolysis when IgG forms complexes with PLG, it was proposed that SAK could be assigned to the staphylococcal virulence factors.

Rooijakkers et al (26) therefore investigated whether SAK had an effect on IgG-mediated phagocytosis of *S. aureus*. For this study, a SAK-negative *S. aureus* strain was used. Using ELISA, IgG bound to the surface of the SAK-negative *S. aureus* strain was detected after being incubated with human serum. However, when bacteria were treated with PLG and SAK, a significant decrease in the amount of IgG was demonstrated. This effect was not seen when bacteria were only treated with PLG or SAK. (Fig. 9A) Since PL hydrolyses IgG, both agents are needed to have an effect on the measured amount of IgG. Since IgG plays a very important role in opsonization of pathogens, it was studied whether treatment with PLG and SAK also had an effect on phagocytosis. For this purspose SAK-negative *S. aureus* was incubated with human IgG and then treated with PLG and SAK. The bacteria were subsequently added to freshly isolated neutrophils, which were given the chance to phagocytose the bacteria. When the bacteria were treated only with PLG, phagocytosis was comparable to control conditions.

However, when the bacteria were treated with PLG and SAK, phagocytosis decreased significantly. (Fig. 9B) (26) Thus, by binding to PLG, SAK inhibits IgG-mediated phagocytosis.

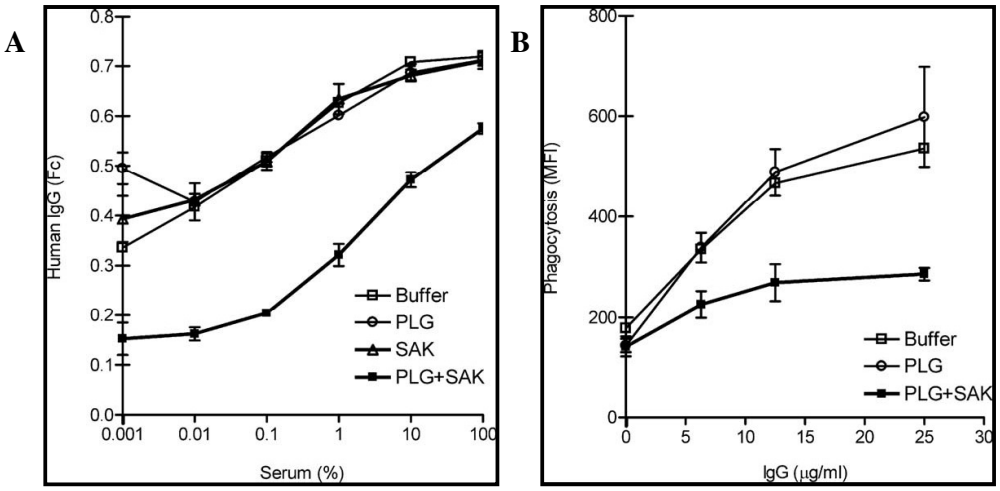

FIGURE 9. Staphylokinase inhibits IgG binding and phagocytosis by neutrophils in the presence of PLG. **A)** Human IgG binding to *S. aureus*. Bacteria were incubated with serum to bind IgG and subsequently treated with buffer, PLG, SAK or PLG + SAK for 2 hours at 37° C. SAK only inhibits IgG binding in the presence of PLG. (26) **B)** The influence of PLG + SAK on Fc receptor-mediated phagocytosis. Fluorescently-labeled *S. aureus* was incubated with 0 – 25 μg/ml purified human IgG and then treated with buffer, PLG or PLG +SAK for 1 hour at 37° C. Bacteria were then added to freshly isolated neutrophils for 15 minutes at 37° C. Fluorescence was then measured in 10.000 neutrophils and represents phagocytosis. SAK only inhibits phagocytosis by neutrophils in the presence of PLG. (26)

Since urokinase, a physiological PLG activator, cleaves of C3b and iC3b into C3d and C3c respectively, it was suggested by Seya et al that SAK could perhaps function as a general opsonin degrader. (27) SAK-negative *S. aureus* was incubated with human serum and treated with PLG and SAK for 2 hours. Using an ELISA essay, human C3b on the treated bacteria was measured. Treating the bacteria with PLG and SAK lead to a significant decrease in the amount of C3b deposited on the surface of *S. aureus*. Once again, this effect could not be seen when only PLG or SAK were used for treatment of the bacteria. (Fig. 10A) To study whether this phenomenon influenced phagocytosis, SAK-negative *S. aureus* was incubated in IgG and IgM deprived human serum. These bacteria were then treated with PLG and SAK for 1 hour and freshly isolated neutrophils were added for 15 minutes. Treatment with PLG and SAK showed a tremendous and significant decrease in phagocytosis, where treatment of only PLG or SAK did not. (Fig. 10B) This demonstrates that SAK is also able to hydrolyze C3b and inhibit complement-mediated phagocytosis in the presence of PLG. (26)

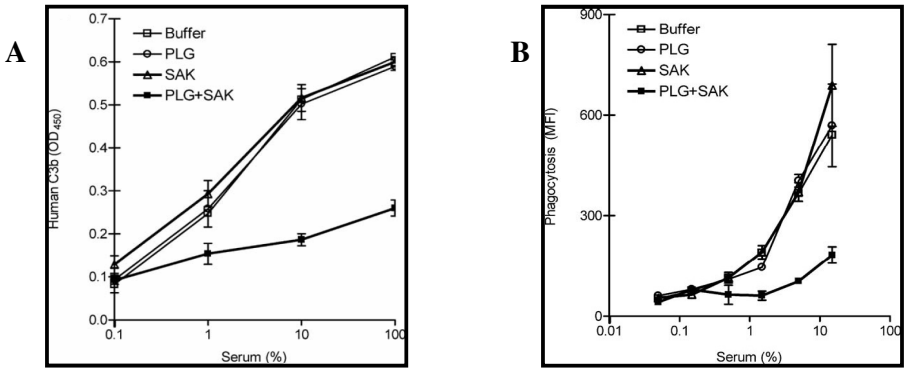

FIGURE 10. Staphylokinase inhibits opsonization by C3b and complement induced phagocytosis by neutrophils in the presence of PLG. **A)** C3b deposition on *S. aureus*. Bacteria were incubated with normal human serum for 20 min. at 37° C. Washed bacteria were then treated with buffer, PLG, SAK or PLG + SAK. C3b deposition was determined using ELISA. C3b deposition on the surface of *S. aureus* was only inhibited in the presence of PLG. (26) **B)** The effect of PLG + recombinant SAK on complement-driven phagocytosis. Fluorescently labeled *S. aureus* was incubated with IgG and IgM derived serum for 20 min at 37° C. Washed bacteria were then treated with buffer, PLG, SAK or PLG + SAK for 1 hour at 37° C and neutrophils were subsequently added for 15 minutes. The fluorescence of 10.000 neutrophils was then measured. SAK inhibits phagocytosis of *S. aureus* only in the presence of PLG (26)

Although nowadays SAK has mainly been described as a protein that is involved in general opsonin degradation, it has also been shown that SAK plays a role in the countering of α-defensin. (24) Defensins are a part of the oxygen-independent mechanisms of leukocytes to fend off pathogens. (12, 16) These agents exert their function by disrupting the pathogen membrane. It has been illustrated that introducing defensins like human neutrophil protein 1 and 2 (HNP-1 and HNP-2) to SAK-negative *S. aureus* shows a significant increase in bacterial killing when compared to SAK-positive *S. aureus* (Fig. 11A). When studying the effect of SAK on HNP-1 and HNP-2, it was found that SAK forms complexes with both HNPs, inhibiting them to efficiently form pores in the bacterium (Fig. 11B).

Comparing treatment of SAK-negative *S. aureus* with combinations of HNP-2, SAK and phenylalanyl-prolyl-arginine-chloromethyl ketone (PPACK) showed that this effect was not caused by activated PLG; PPACK blocks the serine protease domain, thus preventing SAK from binding and activating PLG. It was seen that the bacterial killing of HNP-2 could significantly be decreased by the presence of SAK. Upon introducing PPACK to this mixture, the bacterial killing was not restored. (Fig. 11C). This demonstrates that SAK also binds to HNPs, inhibiting pore formation and thus killing of the bacterium. (24)

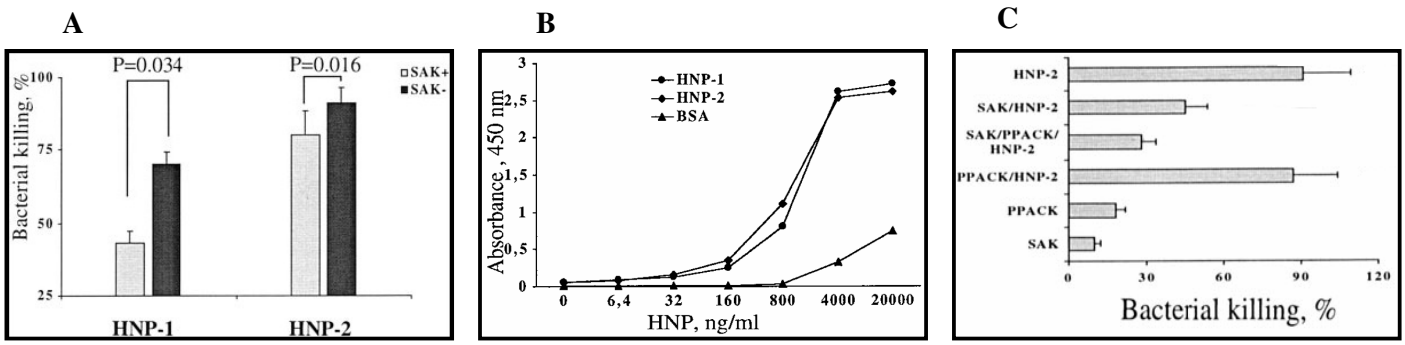

FIGURE 11. Staphylokinase binds to and inhibits bacterial killing by HNP-1 and HNP-2. **A)** Bactericidal effect of α-defensins on *S. aureus* strains depends on their production of SAK. Bacterial growth of several strains was observed with or without the presence of 5 μg/ml HNP-1 or HNP-2. Per strain SAK production was determined after 6 hours of growth. The presence of SAK significantly reduced bacterial killing by both HNPs. (24) **B)** Complex formation between SAK and α-defensins. Wells were coated with recombinant SAK or, as a control, with BSA. HNP-1 or HNP-2 were added, followed by incubation with specific antibodies for an ELISA readout. Binding of SAK to both HNPs is demonstrated. (24) **C)** Inhibition of α-defensins by SAK is independent on its ability to activate plasminogen. Bacterial growth of *S. aureus* strains unable to produce SAK was monitored with the presence of the designated compounds. SAK significantly reduced bacterial killing by HNP-2. Since this also happens in the presence of PPACK this is not established by activating PLG. (24)

2.4 Staphylococcal Complement Inhibitor (SCIN)

SCIN, like SAK, can be found on pathogenicity island 5 in the *S. aureus* genome. (28). Because of its genetic location, SCIN was studied for immunomodulatory activity by Rooijakkers et al. A phagocytosis essay with *S. aureus*, performed in vitro, showed that SCIN blocked bacterial uptake by neutrophils (Fig. 12A). It was also demonstrated that pre-incubating neutrophils with SCIN did not inhibit phagocytosis after removing SCIN and introducing *S. aureus.* This indicated that SCIN did not affect the neutrophils but had an effect on the direct interaction between neutrophils and *S. aureus*. (28) To study whether SCIN was involved in opsonization, phagocytosis induced by purified human IgG was tested. No difference in IgG-enabled phagocytosis was found. (Fig. 12B) However, a significant decrease of C3b deposition was found on the surface of *S. aureus* after incubation in human serum in the presence of SCIN (Fig. 12C). Further studies showed that SCIN was able to prevent C3b deposition on the bacterium in the classical-, the lectin- and the alternate complement pathway. (28) Thus, SCIN is able to prevent opsonization and thereby complement mediated phagocytosis as well.

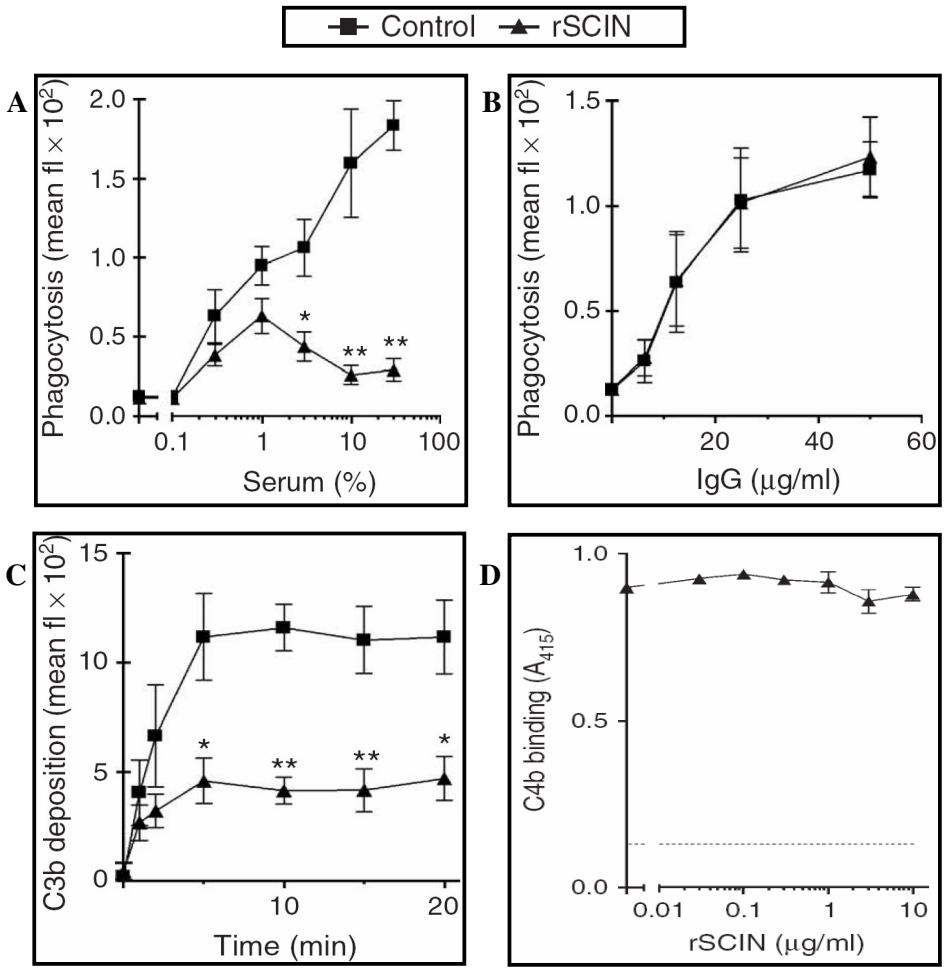

FIGURE 12. Staphylococcal Complement Inhibitor inhibits deposition of C3b on the bacterium and inhibits phagocytosis by neutrophils. **A)** The effect of recombinant SCIN (3µg/ml) on phagocytosis of *S. aureus* by human neutrophils in the presence of serum. *S. aureus* was fluorescently labeled and fluorescence was determined in neutrophil population after phagocytosis. The presence of rSCIN clearly inhibits phagocytosis. (28) **B)** The effect of recombinant SCIN (10µg/ml) on phagocytosis of *S. aureus* by human neutrophils in the presence of purified human IgG. *S. aureus* was fluorescently labeled and fluorescence was determined in neutrophil population after phagocytosis. There is no effect on phagocytosis, demonstrating that rSCIN does not interfere with IgG-driven phagocytosis. (28) **C)** C3b deposition on *S. aureus* in 10% human serum in the presence of rSCIN (3 µg/ml). C3b was detected using fluorescent goat anti-human C3 antibodies. The presence of SCIN causes a significant decrease in C3b deposition on *S. aureus*. (28) **D)** C4b deposition on *S. aureus* in presence of 2% serum. C4b was detected by using fluorescent goat anti-human C4 antibodies. The presence of rSCIN does not affect C4b binding on *S. aureus* in a concentration ranging from 0.01 – 10 µg/µl. (28)

Since SCIN did not bind directly to C3, soluble C3b or membrane bound C3b, interactions with other other components of the complement system were studied (28). It was found that C4b binding was not affected, meaning that SCIN had to act before C3b opsonization but after production of C4b (Fig. 12D). Also, all 3 complement pathways had to be affected. As can be seen in Fig. 3, all 3 complement pathways lead to the initiation of the same enzymes: C3 convertases. There is however a significant difference in the C3 convertases involved in the different pathways; the lectin- and classical pathway result in the generation of the C4b2b complex, also known as the soluble C3-convertase, the alternate pathway results in the generation of the C3bBb complex, also known as the alternative or membrane bound C3 convertase. (10, 11)

During the formation of the soluble C3-convertase, C2 is cleaved into C2a and C2b. C2b forms C3-convertase together with C4b. Upon introduction of zymosan, a yeast cell wall glucan that initiates the lectin pathway, to serum, C2b is formed due to C2 cleavage. (10, 11) In the presence of SCIN however, C2b is not formed (Fig. 13A). During the formation of the membrane-bound C3-convertase, factor B is cleaved into Ba and Bb. Bb forms C3-convertase together with C3b. Upon introducing *S. aureus* to serum, Bb is formed due to factor B cleavage by factor D. Once again however, upon introduction of SCIN Bb will not be formed (Fig. 13B). (28) This demonstrates that SCIN inhibits the formation of C3 convertases, thereby inhibiting the complement cascade.

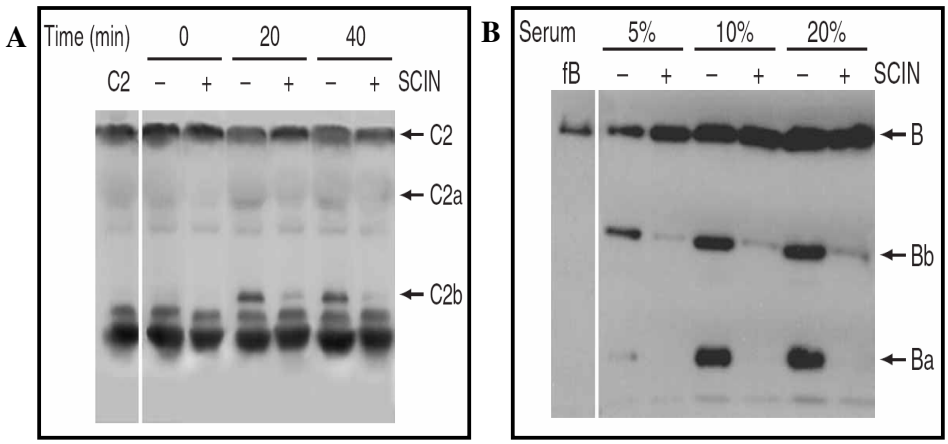

FIGURE 13. Staphylococcal Complement Inhibitor inhibits C2 and factor B cleavage. **A)** Surface detection of C2 split products on zymosan after opsonization in serum plus 10 µg/ml rSCIN. In the presence of rSCIN, a clear decrease in C2a and C2b is demonstrated, showing that the presence of rSCIN inhibits C2 cleavage. (28) **B)** Surface detection of Bb on zymosan after incubation in 20% serum plus 10 µg/ml of rSCIN. In the presence of rSCIN, a clear decrease in Ba and BB can be found, demonstrating that the presence of rSCIN inhibits factor B cleavage. (28)

2.5 Staphylococcal protein A (SpA)

Like SAK, SpA disrupts immunoglobulin opsonization to evade detection by the immune system. SpA is a an extracellular protein, anchored to the cell wall. There is quite some variation in SpA among various *S. aureus* strains and this variation is often used to determine the type of strain that hospitals are dealing with; this is called SpA-typing. (29). Protein A interacts with IgG by binding to its Fc domain. (30) This binding causes the surface of *S. aureus* to become coated with IgG molecules that cannot be recognized by the Fc receptor on neutrophils because they are in the wrong orientation. Analysis of the SpA immunoglobulin-binding module (domain B) by Cedergren et al showed it consists of an anti-parallel three helical bundle, just like the active domains of Efb and SCIN (Fig. 14A). The residues that are involved in the binding of the Fc domain of IgG have been discovered via site-directed mutagenesis (Fig. 14B), demonstrating that SpA is able to bind human IgG.(31)

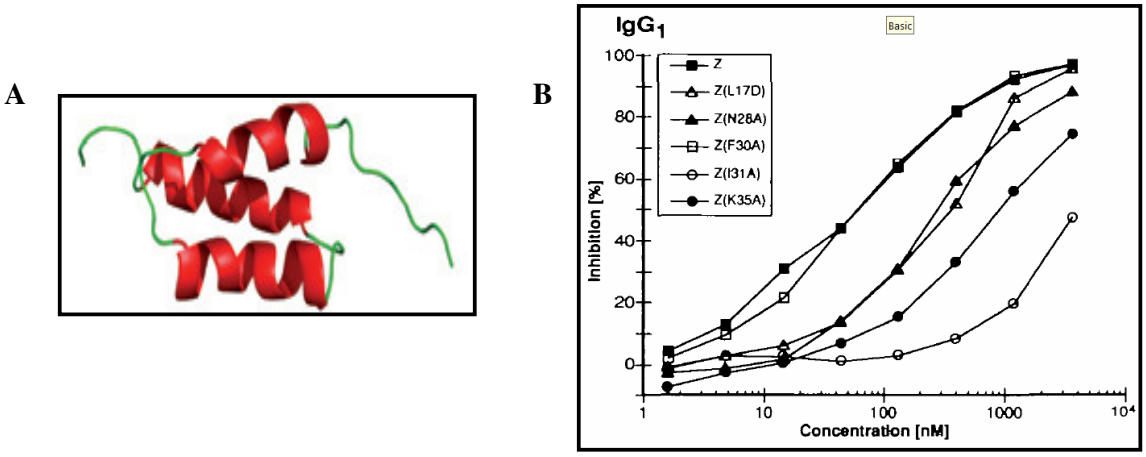

FIGURE 14. Staphylococcal Protein A binds to human IgG. **A)** The immunoglobulin-binding module (domain B) of Staphylococcal Protein A. It consists of a three helical bundle. (10) **B)** Inhibition of binding of a functional analog of Protein A domain B (Z) to human monoclonal IgG_1, demonstrating that SpA does indeed bind to immunoglobulin (31).

Coating of the bacterium decreases complement opsonization indirectly: since the bacterium is covered with incorrectly orientated IgG, C3b will not be able to bind to the bacterium. Thus, C3 convertases will not be able to form and the remaining complement cascade will not take place. (10, 11) Carbohydrates are also more difficult to reach by MBL or folins, inhibiting the lectin pathway as well. Furthermore it has been demonstrated that SpA binds to the ubiquitously distributed cellular protein gC1qR/p33; a binding site for C1q. (31) This is thought to be more of a mechanism for bacterial cell adhesion however, then being involved in immune evasion. Recent findings also illustrate that SpA can bind to the Von Willebrand factor (involved in platelet adhesion to wound

sites) and Tumor Necrosis Receptor 1 / CD120 (involved in apoptosis and immune regulation). (32) It has therefore been proposed that SpA might play a more elaborate role during infection then is currently assumed.

2.6 Staphylococcal superantigen-like 7 (SSL7)

As can be seen for and SpA, SSL7 evades the acute immune response by interacting with immunoglobulins. Like in SAK however, it evades the human immune system by interacting with the complement system as well. SSL7 is a member of the the family of SSLs, which are named after their homology to the staphylococcal super-antigens. Unlike super-antigens, the domains important for T-Cell Receptor and MHC Class II binding are not conserved and they therefore do not induce the same drastic non-specific T-cell response. Since SSLs occur in all *S. aureus* strains, it is believed that they play a key role in bacterial survival. (33, 34) Since the SSLs are located on *S. aureus* Pathogenicity Island 2, it was hypothesized that they play a role in targeting the host immune system like most proteins situated on *S. aureus* Pathogenicity Island 2. (33)
When Langley et al studied what proteins from human serum SSL7 would bind to, they found 4 peptides: the Light and Heavy chain of Immunoglobulin A (IgA), C5a and C5b (Fig. 15A). Since the presence of IgA could also indicate that the human serum that was used contained antibodies against SSL7, the serum of 14 individuals was tested with the same assay (Fig. 15B). In 13 out of 14 people, the amount of IgA acquired was consistently strong. This demonstrates that SSL7 targets IgA rather then vice versa, since the amount of IgA directed against SSL7 would vary among individuals. (35)

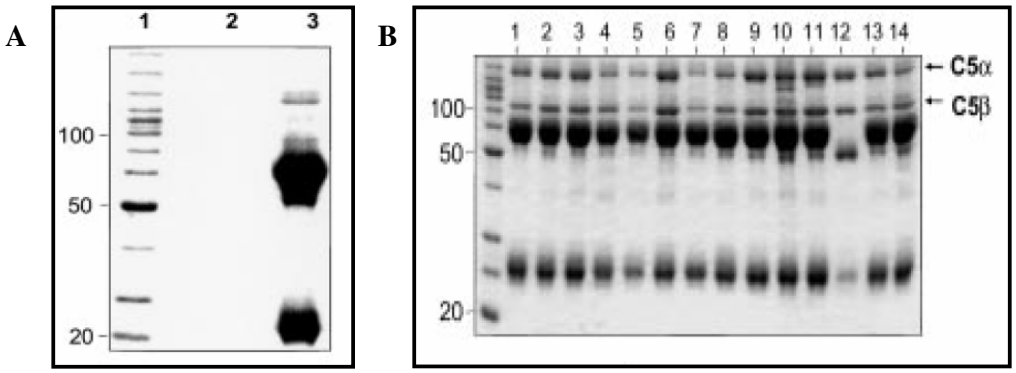

FIGURE 15. Staphylococcal Superantigen-like protein 7 binds to human IgA and C5. **A)** Serum proteins bound by SSL7. SSL7 bound to agarose beads was incubated with serum for 1 hour at 4° C whilst rotating. The proteins bound to the SSL7 covered agarose beads were isolated and run on an SDS-page gel. Recombinant SSL7 binds proteins from human serum (lane 3), but not from fetal calf serum (lane 2). (35) **B)** SSL7 bound proteins from 14 human volunteers. Experiment was performed according to the same protocol as was used in Fig. 15A. Except for individual 12, the acquired amount of IgA (60-kD) is consistently strong. In all individuals, C5a and C5b are also acquired in consistent amounts. is barely any variation among individuals (except nr. 12, who turned out to have a genetic IgA deficiency) in the amount of bound protein, demonstrating that SSL7 binds IgA, rather then IgA binding SSL7. (35)

Subsequently, Hair et al studied whether the presence of SSL7 also interfered with the binding of the IgA to its receptor, FcαRI. Using a recombinant, soluble form of FcαRI, the effect of SSL7 on the binding of IgA to FcαRI was studied (Fig. 16A). In a dose-dependent fashion, the binding of IgA to FcαRI decreased as a result of SSL7. This data suggests that SSL7 could prevent leukocyte activation by IgA opsonization. Unfortunately it was not possible to test this in vivo, since SSL7 does not react with mouse IgA. (35) Since C5b plays an important role in the formation of the Membrane Attack Complex, it was hypothesized that the presence of SSL7 could also prevent the killing of bacteria by the complement system. (10, 11). Since no *S. aureus* SSL7 knockout strain was available, *E. coli* was studied in presence of SSL7. (Fig. 16B). It was demonstrated that pretreating human serum with SSL7 before adding *E. coli* to the serum, up to 14% of the *E. coli* could be saved, compared to 0% untreated serum. (35) In conclusion this data demonstrates that SSL7 binds to IgA, C5a and C5b and inhibits killing of bacteria by human serum. Since no SSL7 knockout of *S. aureus* has been discovered, direct effects of SSL7 on MAC formation, leukocyte chemotaxis or phagocytosis have not yet been demonstrated.

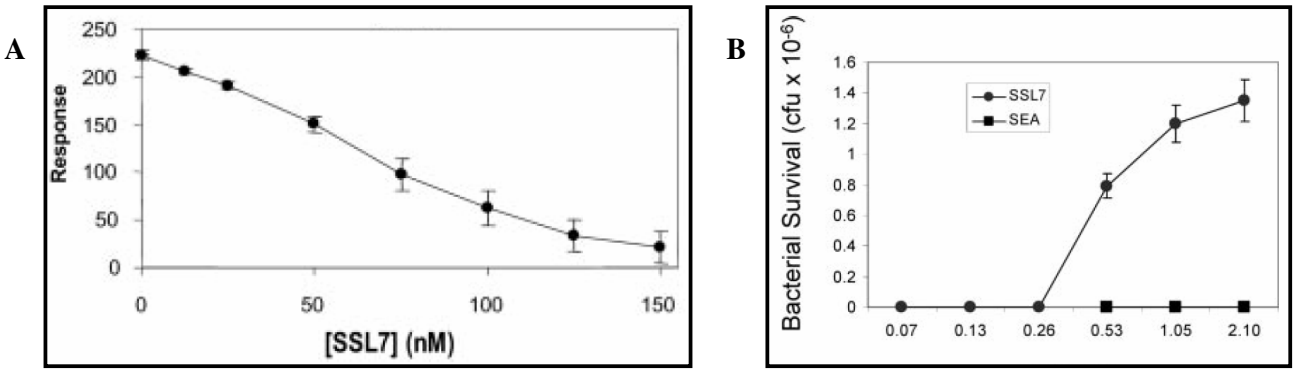

FIGURE 16. Staphylococcal Superantigen-like protein 7 inhibits binding of IgA to FcαRI and inhibits bacterial killing by the complement system in *E. coli*. **A)** Dose-dependent inhibition by SSL7 on IgA binding to rsFcαRI. Data was acquired using Biacore. Data showed that in the presence of SSL7, there is a dose-dependent decrease of IgA binding to rsFcαRI. (35) **B)** Recombinant SSL7 increases bacterial survival in serum. Human serum was incubated with SSL7 or SEA (negative control, which is highly homologous to SSL7) (33) from 30 minutes at 37° C. 10^7 *E. coli* were then added to the serum. The amount of bacteria that survived was determined by plating on agar and scoring of colonies. SSL7 is shown to inhibit *E. coli* killing by human serum (and thus, complement) in a dose-dependent manner, whereas SEA is not. (35)

Chapter 3 Neutrophil evasion

Neutrophils are often the first immune cells at the site of infection and are very proficient in the killing of bacteria. Therefore, it is not surprising that another large amount of agents that *S. aureus* employs to evade the host immune system are aimed at the evasion of neutrophils. The most important proteins and strategies used by *S. aureus* that are involved in neutrophil evasion will be discussed in this chapter.

3.1 Extracellular Adherence Protein (Eap)

One of the first proteins described in *S. aureus* involved in neutrophil evasion is Eap. This 60-kD protein was discovered in 1993 and displayed a very broad array of binding activities. Next to being named Eap, this protein was named MHC Class II Analogous Protein (MAP) as well. (36) This protein is excreted, but small amounts can also be found on the surface of *S. aureus* due to binding to the bacterium. Similar to Efb and ClfA, at first it was believed that the main function of this protein was to aid *S. aureus* in infecting damaged tissues by binding to fibrinogen, fibronectin and vitrinogen; components of the extracellular matrix. (36) Almost 10 years passed before someone decided to thoroughly investigate whether Eap could also interact with the host immune system. Since other proteins in the *S. aureus* genome were also able to bind to fibrinogen, McGavin et al studied if Eap was also able to bind to immunomodulatory proteins that are near or in the Extracellular Matrix. It was hypothesized that it would most likely interact with "different extracellular and/or cell surface-associated adhesion proteins" such as integrins. (37) Blocking the binding of LFA-1, Mac-1 and VLA-4 (utilized by neutrophils) to their endothelial counterparts ICAM-1, ICAM-2 and VCAM-1 respectively, would block extravasation of neutrophils towards the site of infection. (13)
As was shown in earlier research, Eap inhibited the binding of fibrinogen and vitonectrin to their ligands (Fig. 17A and 17B). When this same experiment was performed with ICAM-I it was shown that the presence of Eap also inhibited the binding of ICAM-I to LFA-1 (Fig. 17C, white bars) as well as the binding of ICAM-I to Mac-1 (Fig 17C, black bars). (37)

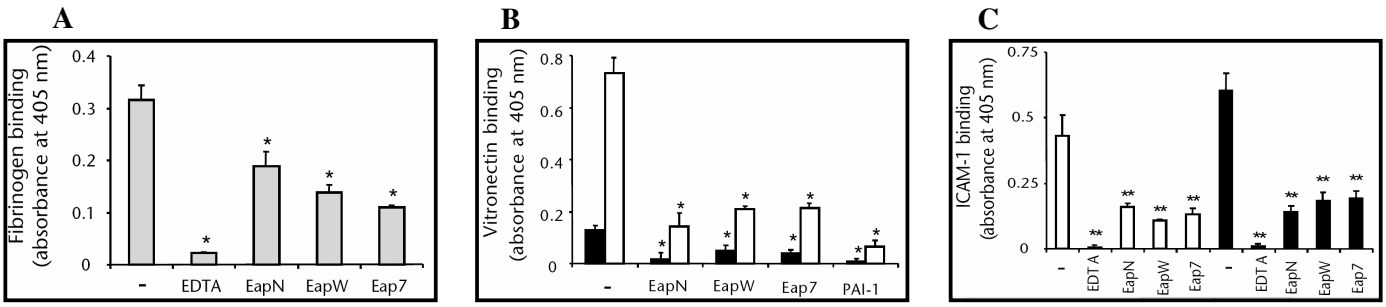

FIGURE 17. Extracellular adherence protein inhibits binding of fibrinogen and vitonectrin to Mac-1 and LFA-1 or Mac-1 to ICAM-1. **A)** Interference of binding of fibrinogen to Mac-1 in the presence of EDTA or Eap from 3 different *aureus* strains. Fibrinogen – Mac-1 binding was determined using ELISA. The presence of all 3 types of Eap significantly decrease binding of fibrinogen to immobilized Mac-1. (36). **B)** Interference of binding of vitonectrin to Mac-1 in the presence of EDTA or Eap from 3 different *aureus* strains. Vitonectrin– Mac-1 binding was determined using ELISA. The presence of all 3 types of Eap significantly decreased binding of vitonectrin to immobilized Mac-1. Presence of urokinase plasminogen (black bars) abolishes the effect of Eap. (36). **C)** Binding of LFA-1 (white bars) or Mac-1 (black bars) to ICAM-1. Binding was determined using ELISA. Immobilized LFA-1 or Mac-1 significantly lost their ability to bind to ICAM-1 in the presence of EAP. (36)

It was subsequently shown by Chavakis et al that Eap bound to ICAM-I with high affinity and that Eap did not bind to Mac-1 or LFA-1. (37). It was subsequently demonstrated that after inducing inflammation, neutrophil migration to inflamed tissue in vivo could be inhibited by injecting tissue with Eap (Fig. 18A). This was also demonstrated when the Newman strain (wildtype) or the AH12 (Eap deficient) strain were used to induce inflammation of the tissue (Fig. 18B). It was shown that the AH12 strain attracted a significantly higher amount of neutrophils to the site of infection. Administering antibodies against ICAM-I before the infection showed a decrease in the amount of neutrophils attracted to the site of infection to such an extent that there was no longer a significant difference in both strains. (37) Thus, expression of Eap inhibits migration of neutrophils to tissue infected with *S. aureus* by inhibiting the binding of ICAM-I to LFA-1.

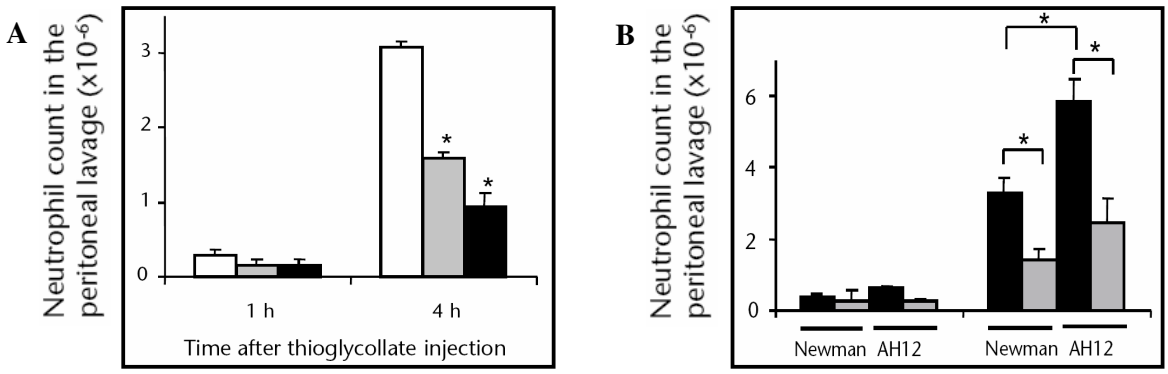

FIGURE 18. Extracellular adherence protein as well as the presence of *S. aureus* inhibit neutrophil migration in acute inflammation in vivo. **A)** Inhibition of neutrophil migration by Eap in acute inflammation caused by thioglycollate in vivo. 1 and 4 hours after injection in to the peritoneum, the neutrophils in the peritoneal lavage are determined. Before infection mice were treated with PBS (white bar), a blocking mouse-antibody against ICAM-1 (black bar) or Eap (gray bar). A significant decrease can be seen in the amount of neutrophils present at the site of infection 4 hours after injection if pre-treated with a mouse-antibody against ICAM-1 or Eap. (36) **B)** Inhibition of neutrophil migration by Eap in acute inflammation caused by presence of *S. aureus* in vivo. Experiment was performed in the same manner as in Fig. 18A except infection was now induced by injecting *S. aureus* or an Eap-deficient *S. aureus* strain (AH12) in to the peritoneum. 30 minutes before treatment, mice were injected with buffer (black bars) or with a blocking mouse antibody against ICAM-I (gray bars). The Eap-deficient *S. aureus* strains attracts significantly more neutrophils then the wildtype *S. aureus* strain. Pre-treatment with antibodies against ICAM-I significantly causes a decrease in neutrophil attraction for both strains, indicating that neutrophil attraction occurs according to the same mechanism for both strains. (36)

As was suggested for ClfA it was hypothesized that as a result of binding to fibrinogen, Eap could induce internalization by host cells. (38) To study this, Haggar et al compared adherence of the *S. aureus* Newman and AH12 strains to fibroblast and epithelial cells. (Fig. 19A). A decrease in adherence was demonstrated for the Eap deficient *S. aureus* strain AH12. Subsequently, the amount of internalized bacteria was measured (Fig. 19B). It was consequently shown that there was also a significant decrease in internalization of *S. aureus* in the Eap deficient strain. Further studies showed that internalization of *S. aureus* by fibroblast cells was significantly increased in the presence of Eap (Fig. 19C). (8) Haggar et al state that Eap induces both adherence and internalization. However, even though an increase in internalization in the presence of Eap was shown, since no correction was applied for the decrease in adherence when internalization was studied these data are not fit to conclude that Eap is directly involved in processes other then adherence.

In conclusion: Eap is a versatile protein that binds to components of the Extracellular Matrix, which could play an important role in colonization of e.g. open wounds (8). Furthermore it is shown to disrupt neutrophil migration to inflamed tissue and increases in vitro adherence to fibroblast and epithelial cells which induces internalization, an important strategy to elude the host immune system.

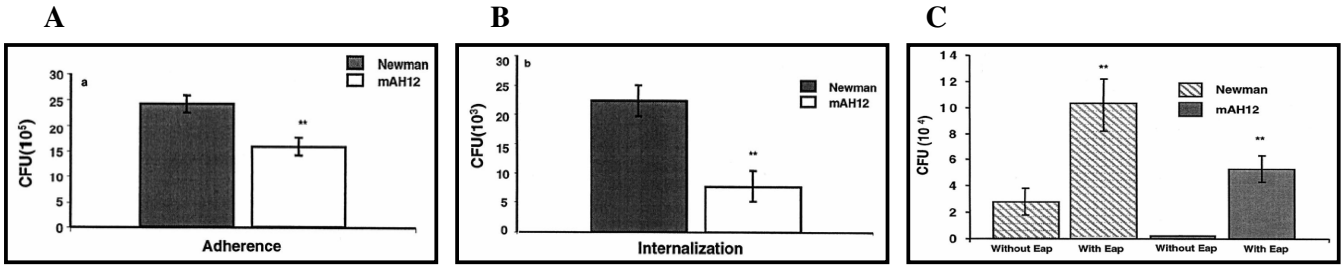

FIGURE 19. Extracellular adherence protein expression in *S. aureus* induces binding to and internalization by fibroblast cells. **A)** Adherence of wildtype *S. aureus* (Newman) and Eap-deficient *S. aureus* (mAH12). A confluent layer of fibroblasts was inoculated with the *S. aureus* strains and incubated at 37° C for 2 hours. The fibroblast cells were then washed. *S. aureus* was isolated and plated out in dilutions to determine the amount of *S. aureus* that adhered to the fibroblast cells. Eap-deficient *S. aureus* shows a significant decrease in adherence to fibroblast cells when compared to the wildtype strain. (8) **B)** Internalization of wildtype *S. aureus* (Newman) and Eap-deficient *S. aureus* (mAH12). The experiment was performed in a similar manner as described in Fig. 19A but after the washing of the cells the cells were incubated with lysostaphin, killing all *S. aureus*. Fibroblast cells were then lysed and contents were plated out in dilutions to determine the amount of *S. aureus* that was internalized by the fibroblast cells. Eap-deficient *S. aureus* is significantly less internalized by fibroblast cells when compared to the wildtype strain. (8) **C)** Internalization in the presence of externally added Eap. The experiment was performed in a similar manner as described in Fig. 19B but in this experiment Eap was also added. In both strains, addition of Eap shows a significant increase in internalization of *S. aureus* by fibroblast cells. In the wildtype strain that already expresses Eap the presence of additional Eap increases internalization even further. (8)

3.2 Chemotaxis Inhibitory Protein of *Staphylococcus aureus* (CHIPS)

Like Eap, CHIPS inhibits chemotaxis in neutrophils towards sites of infection. It is located alongside the gene coding for SAK in the *S. aureus* genome. CHIPS was the first protein found to block the binding of C5a, as well as the binding of formylated peptides from *S. aureus* to neutrophil receptors (3). The study that led to the discovery of CHIPS was a follow-up on a study showing that *S. aureus* supernatant (SaS) disrupts neutrophil activity. In this study it was demonstrated that pretreatment of neutrophils with SaS blocked binding of labeled antibodies to C5a and formylated Met-Leu-Phe (fMLP) receptors (Fig 20A). It was therefore hypothesized by Veldkamp et al that there were one or more compounds in SaS that inhibit interaction between these receptors and their ligands. (39) Since stimulation of C5a receptors and fMLP receptors induces chemotaxis and stimulation fMLP receptors induces degranulation as well the effect of CHIPS on receptor activity was studied. (12) Calcium flux was measured since all chemokine receptors are known to induce a calcium influx in neutrophils quite rapidly after they are stimulated with their respective ligands (16). When stimulating both receptors with their respective ligands, a concentration-dependent decrease in calcium flux was demonstrated in the presence of SaS (Fig. 20B). Using transwell systems, the effect of migration towards C5a and fMLP was studied, showing that SaS inhibited neutrophil migration towards both chemoattractants. (Fig. 20C). (39)

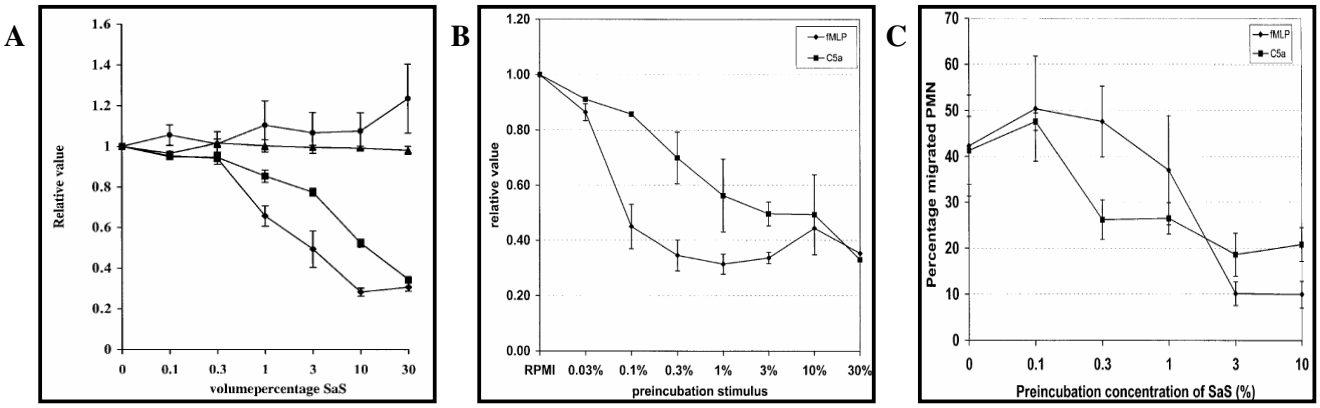

FIGURE 20. *Staphylococcus aureus* supernatant decreases binding availability in C5a-R and fMLP-R, inhibiting chemotaxis and calcium responses to C5a and fMLP. **A)** Chemokine receptor binding availability on neutrophils under influence of SaS. Neutrophils were incubated in SaS for 30 minutes. Data is expressed in a relative manner in relation to neutrophils that were not incubated in SaS. Binding was studied by introducing receptor-specific fluorescent antibodies to 5000 neutrophils and measuring fluorescence. ◆= fMLP-R ● = Paf-R ■ C5a-R▲ = IL-8-R. A large decrease in binding availability is demonstrated for C5a-R and fMLP-R. (39) **B)** Calcium flux in neutrophils. Neutrophils were loaded with 2 μM Fluo-3-AM and washed. The cells were then stimulated with fMLP or C5a. Calcium response was measured within 10 seconds after stimulation by measuring fluorescence. Pre-incubation with SaS inhibited response to fMLP and C5a in a dose-dependent manner. (39) **C)** Chemotaxis in neutrophils. Using Transwell-systems, fluorescently labeled neutrophil migration was studied under the influence of C5a and fMLP after pre-treatment with SaS. After 30 minutes the fluorescence in the bottom well was measured. Pre-treatment with SaS significantly decreases neutrophil chemotaxis towards C5a and fMLP in a dose-dependent manner. (39)

De Haas et al discovered a 14.1-kD protein that showed the chemotaxis-inhibiting properties for fMLP and for C5a, as well as the ability to inhibit the binding of labeled fMLP and labeled C5a to neutrophils (Fig. 21A, black circles). The protein was named CHIPS, the gene was named *chp*. (40) Using a CHIPS knockout strain it was demonstrated that this immunomodulating activity on neutrophils could be abolished by deleting *chp* (Fig. 21A, white circles). Subsequently it was shown that adding a plasmid with the *chp* gene, including its promoter region, to the knockout strain restored the immunomodulating activity of the supernatant of the knockout strain (Fig. 21A, squares), demonstrating that CHIPS was responsible for this activity. Furthermore, in vivo studies in mice showed that migration of neutrophils to tissue after injecting C5a was inhibited by pre-injecting the tissue with CHIPS (Fig. 21B). It must be stated however that human C5a was used since CHIPS has a decreased effectiveness of approximately 30 times in mice. (40)

Since CHIPS only inhibits the receptors for C5a and fMLP, it does not fully block neutrophil migration. For instance, it is not able to block chemotaxis towards IL-8. It is therefore believed that CHIPS plays a more important role in initial colonization then it does in persistent infections, (data not shown) (40). Interestingly, like SEA and SAK, CHIPS seems to be human specific. Since these proteins are all on the same bacteriophage it seems likely that the presence of this bacteriophage in the genome of an *S. aureus* strain gives rise to an advantage for the infection of humans. (28)

In conclusion, CHIPS blocks the binding of C5a and fMLP to their receptors, decreasing activation as well as chemotaxis by these ligands in neutrophils. Furthermore it inhibits neutrophil chemotaxis towards tissue colonized by *S. aureus* in in vitro experiments.

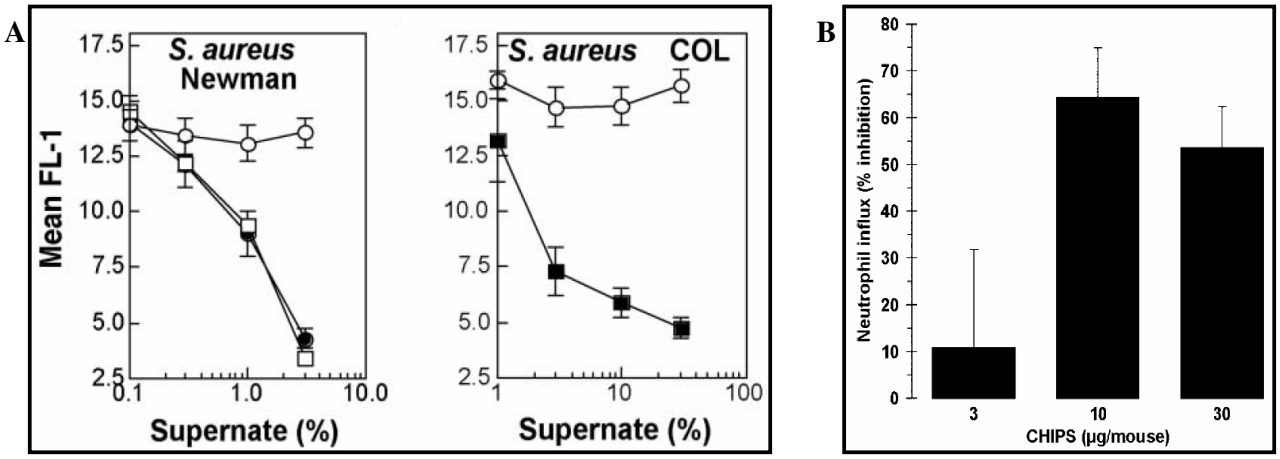

FIGURE 21. Chemotaxis inhibitory protein inhibits fMLP binding to neutrophils in vitro inhibits C5a chemotaxis in vivo. **A)** Binding of labeled fMLP to neutrophils. Neutrophils were pre-treated with different concentrations of SaS. Black circles: wildtype *S. aureus*, White circles: Δ*chp S. aureus*, White squares: Δ*chp S. aureus* transformed with a plasmid containing *chp*, Black squares: *S. aureus* COL (naturally occurring *S. aureus* strain not possessing *chp*) transformed with a plasmid containg *chp*. The supernatant of the wildtype strain containing *chp* caused a significant decrease in fMLP binding to neutrophils. This effect can be abolished by deleting *chp* and restored by transforming the Δ*chp* strain with a plasmid containing *chp*. Supernatant from a wildtype strain that does not possess *chp* has no effect on fMLP-binding to neutrophils. If this strain is transformed with a plasmid containing *chp* however, a significant decrease in fMLP binding by neutrophils can be seen. This demonstrates that *chp*, and therefore CHIPS, is responsible for the inhibiting effect of SaS on fMLP binding to neutrophils. (40) **B)** Inhibition of C5a-mediated neutrophil migration in vivo. Mice pre-treated with different concentrations of CHIPS were injected in the peritoneum with C5a 15 minutes later. After 3 hours the influx of neutrophils in the peritoneal lavage was analyzed. Data are expressed as inhibition compared to control-mice in percentages. A decrease of neutrophil influx of up to 65% can be demonstrated by pre-treating the mice with CHIPS. This shows that CHIPS can inhibit C5a chemotaxis in vivo. (40)

3.3 Staphylococcal super antigen-like 5 (SSL5)

After the discovery of CHIPS, further studying showed that the C-terminal region of CHIPS was highly homologous to the C-terminal domains of SSL5 and SSL7. Therefore, Bestebroer et al studied if SSL5 would bind to different populations of leukocytes (Fig. 22A). (34) High binding of SSL5 to monocytes, neutrophils and NK cells was demonstrated. Subsequently, a selection of receptors from these cells was made and the effect of SSL5 to binding of specific antibodies to these receptors was studied. Of approximately 30 receptors that were tested, only PSGL-1 showed a decrease in antibody binding in the presence of SSL5. Consequently, this effect was only demonstrated on PSGL-1 present on neutrophils, monocytes and NK cells, not on PSGL-1 on T lymphocytes (PSGL-1 is not expressed in B lymphocytes). PSGL-1, as mentioned before, binds P-selectin which is expressed on the surface of endothelial cells. This is vital for the mediation of rolling of neutrophils on epithelial cells (12). Further studying showed that SSL5 inhibited binding of 3 antibody's to PSGL-1 (PL1, Pl2, KPL1) in a dose-dependent fashion (Fig. 22B). Since SSL5 contained a region homologous to that of CHIPS, an antibody direct against the receptor for C5a was tested as well (W17/1). However, no inhibition in binding of C5a to C5aR was demonstrated.

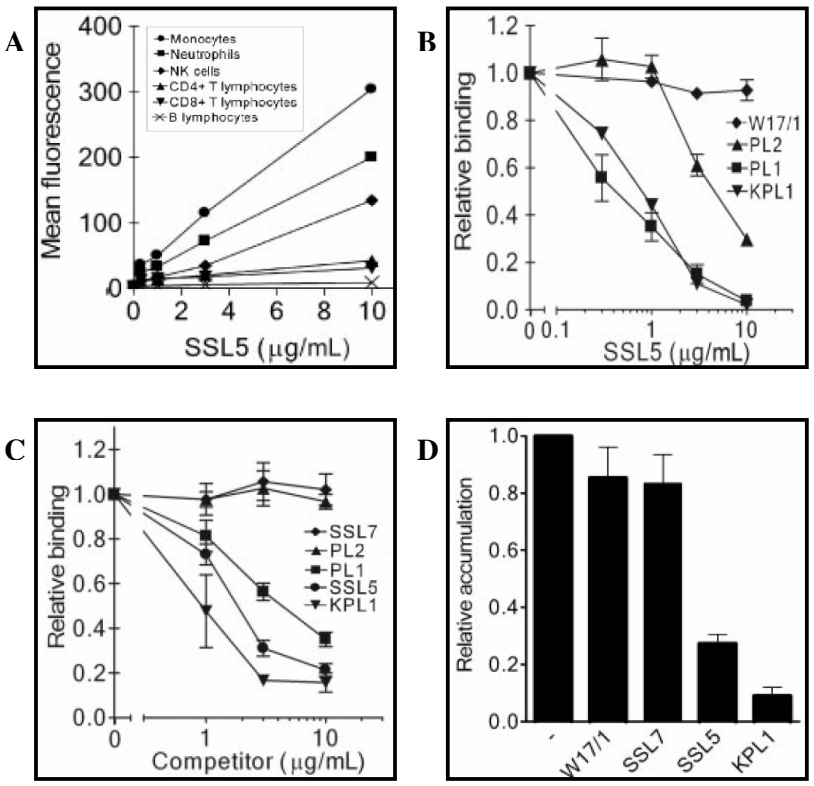

FIGURE 22. Staphylococcal superantigen-like 5 binds to PSGL-1 in monocytes, neutrophils and NK cells, inhibiting PSGL-1 binding to P-selectin and neutrophil rolling. **A)** Binding of labeled SSL5 to leukocytes. Leukocytes were incubated with the labeled SSL5 for 30 minutes on ice. Cell-types were determined using leukocyte-subset-specific antibodies. SSL5 binds to monocytes, neutrophils and NK cells, but not to B- or T lymphocytes. (34) **B)** Anti-PSGL-1 mouse antibodies binding to neutrophils. Neutrophils were pre-treated with SSL5 for 30 minutes on ice. After washing, mice were treated with anti-PSGL-1 antibodies. Binding of these antibodies was determined using labeled goat-anti mouse IgG. SSL5 pre-treatment inhibited binding of all PSGL-1 directed antibodies, but did not inhibit W17/1, an anti-C5aR antibody. (34). **C)** Competition of SSL5 with P-selectin/FC binding to CHO-PSGL-1 cells. Cells were incubated with SSL5, SSL7 or anti-PSGL-1 mouse antibodies for 30 minutes on ice. Cells were then treated with P-selectin/Fc, of which binding was detected with labeled goat anti-human IgG. The values are relative to binding to control-treated cells. Presence of SSL5 and 2 out of 3 anti-PSGL-1 antibodies reduced binding up to 5-fold. (34) **D)** Effect of SSL5 on rolling adhesion of neutrophils. Human umbilical vein epithelial cells were cultured on glass coverslips, treated with SSL5, SSL7, W17/1 (C5aR antibody) and KPL1 (PSGL-1 antibody), and were then perfused. When compared to control-treated cells, presence of SSL5 caused a decrease of rolling adhesion over 70%. (34)

Using Chinese Hamster Ovary (CHO) cells that expressed PSGL-1, Bestebroer et al demonstrated that SSL5 inhibited binding of P-selectin to PSGL-1 (Fig. 22C). When compared to untreated cells, relative binding decreased in a dose-dependent fashion up to 5-fold in the presence of SSL5. Comparable effects were demonstrated using PL1 and KPL1. SSL7 and PL2 did not inhibit PSGL-1 binding to P-selectin.
Finally, using freshly isolated human umbilical vein epithelial cells, the effect of SSL5 on neutrophil rolling was measured (Fig. 22D). When compared to control-treated cells, a decrease of rolling adhesion of over 70% was demonstrated in the presence of SSL5, showing that SSL5 hampers the rolling of neutrophils on endothelial cells in vitro. (34)

Bestebroer et al also studied the effect of SSL5 on calcium-flux in neutrophils, by pre-treating them with SSL5 and subsequently stimulating with several lipid-, protein- or bacteria derived stimuli (Fig. 23A). This showed that, next to the hampering of rolling of neutrophils, SSL5 also inhibited activation of neutrophils when stimulated with C3a, C5a and IL-8 (CXCL8). Other CXC chemokines (chemokines where the two N-terminal cysteines are separated by one amino acid) were tested as well and in all CXC tested chemokines SSL5 elicited the same response (41). Since activation of neutrophils leads to rapid actin polymerization to induce mobilization, the effect of SSL5 on actin polymerization induced by IL-8 was studied using fluorescent phallacidin, demonstrating that treatment with SSL5 inhibited actin polymerization as a result of activation by IL-8 (Fig 23B). (12, 41) SSL5 also inhibited actin polymerization as a result of stimulation with C5a and did not affect polymerization as a result of fMLP (not shown). Using transwell systems, chemotaxis towards IL-8 was investigated (Fig. 23C). After 30 minutes, SSL5 reduced chemotaxis towards IL-8 in neutrophils up to 50%. SSL5 had no effect on chemotaxis towards fMLP in neutrophils (data not shown), demonstrating that SSL5 significantly inhibits chemotaxis towards IL-8. (41) Furthermore it was shown that SSL5 was no longer able to inhibit calcium mobilization in neutrophils after being treated with neuraminidase, demonstrating that SSL5 required glycan-dependent binding to exert its effect. Subsequently it was demonstrated that SSL5 binds to the N-terminus of G protein-coupled receptors in a glycan-dependent manner. G protein-coupled receptors have glycosylation sites at their N-terminus which are believed to be essential for the recognition of their ligands. Using antibodies directed against the N-terminal domain of C5aR, the inhibiting effect of stimulation in presence of C5a was abolished. (34, 41)
Thus, SSL5 inhibits rolling and tethering of neutrophils by inhibiting binding of PSGL-1 to P-selectin, inhibits activation of neutrophils by inhibiting binding of CXC chemokines to their receptors, inhibits activation of neutrophils by C3a and C5a and inhibits chemotaxis towards IL-8.

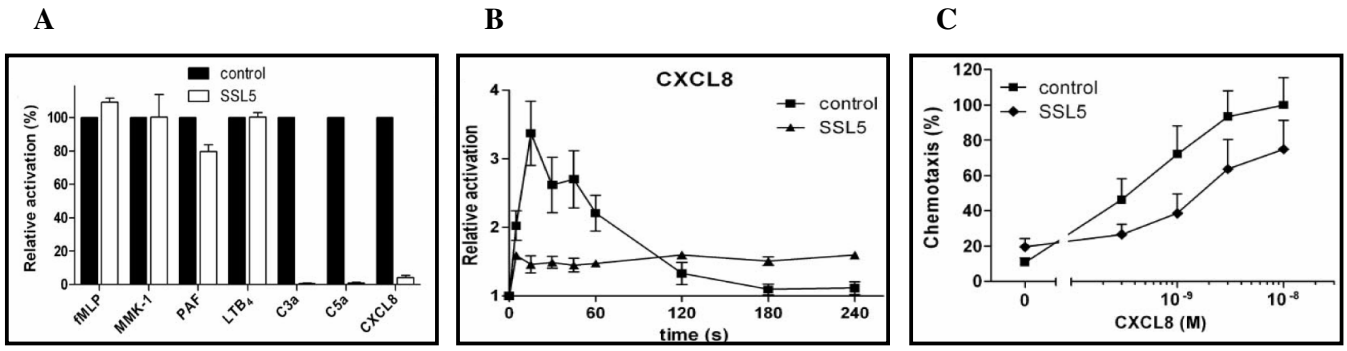

FIGURE 23. Staphylococcal superantigen-like 5 inhibits neutrophil activation by C3a, C5a and IL-8 and inhibits chemotaxis towards IL-8. **A)** SSL5 inhibition of neutrophil activation by chemokines. Neutrophils were pre-treated with SSL5 or buffer for 30 seconds and then stimulated with several chemokines. Calcium flux was measured in the same manner as described in Fig. 20B. Pre-treatment with 10 µg/mL SSL5 abolishes neutrophil activation by C3a, C5a and CXCL8. (41). **B)** The effect of SSL5 on actin-polymerization after activation by CXCL8. Neutrophils were pre-treated with 30 µg/mL SSL5 for 60 seconds and then stimulated with CXCL8. Actin polymerization in neutrophils was measured using labeled phallacidin, which binds to activated actin. If untreated with SSL5 immediate actin polymerization is seen which lasts for approximately 2 minutes. This effect is abolished by pre-treatment with SSL5. (41) **C)** The effect of SSL5 on neutrophil migration towards CXCL8. Using Transwell membranes and labeled neutrophils, chemotaxis towards CXCL8 was measured after 30 minutes. In the presence of 10 µg/mL of SSL5, chemotaxis is significantly decreased. (41).

3.4 FPRL1 Inhibitory Protein (FLIPr)

Like in SSL5, the search for genes showing homology to CHIPS also lead the discovery of FLIPr. The gene for this protein, *flr*, was found in approximately 60% of *S. aureus* isolates and, due to its homology to CHIPS, was believed to be involved in the evasion of the immune system. (42) Since CHIPS inhibits leukocyte activation by blocking the receptors for C5a and fMLP, the effect of FLIPr on neutrophil activation by C5a and fMLP was investigated by Prat et al. (40)

By measuring calcium influx, the stimulation of neutrophils by fMLP in the presence of CHIPS was investigated (Fig. 24A). A significant inhibition of stimulation by FLIPr was shown, however the demonstrated inhibition was significantly less then the inhibition demonstrated by CHIPS. Since FLIPr displayed a partial inhibition it was hypothesized that FLIPr was perhaps able to affect the Formyl Peptide Receptor Like receptor 1 (FPRL1). The effect of FLIPr and CHIPS on binding of FPRL1 to several FPRL1 antagonists was investigated. 3 peptides, (WKYMVm, WKYMVM and MMK-1) which induce a calcium-response in neutrophils as well as in monocytes were used. CHIPS did not inhibit neutrophil nor monocyte stimulation with these peptides. FLIPr however inhibited stimulation significantly, especially in MMK-1 (Fig. 24B and 24C). (42)

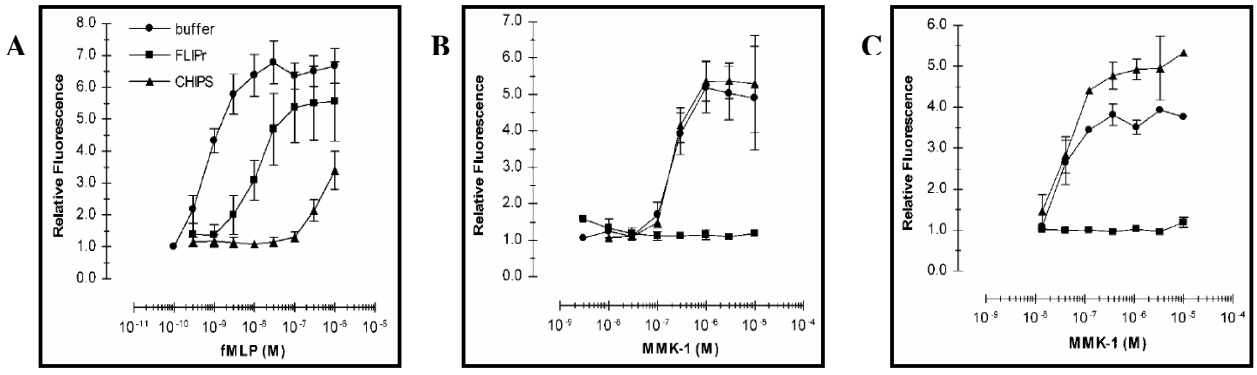

FIGURE 24. FPRL1 Inhibitory Protein inhibits fMLP induced calcium-flux in neutrophils and FPRL1 agonist induced calcium-flux in neutrophils and monocytes. **A)** FLIPr inhibits FPR agonist-induced calcium-flux in neutrophils. Neutrophils were pre-treated with 3 µg/ml buffer, FLIPr or CHIPS, washed and then activated with fMLP. Calcium flux was measured in a similar manner as in Fig. 20B. As shown before, CHIPS effectively inhibits calcium flux in neutrophils after stimulation with fMLP. FLIPr is also able to do this to a lesser, but still significant extent. (42). **B)** FLIPr inhibits FPRL1 agonist induced calcium-flux in neutrophils. The experiment was performed in the same manner as was explained for Fig. 22A, but now the neutrophils were stimulated with MMK-1, an FPRL1 agonist. FLIPr drastically reduces the calcium flux in neutrophils as a response to MMK-1, whereas CHIPS is not. (42) **C)** FLIPr inhibits FPRL1 agonist induced calcium-flux in monocytes. The experiment was performed in the same manner as was explained for Fig. 24B, but now monocytes are stimulated. The same result can be seen here as was seen for neutrophils: FLIPr drastically reduces the calcium flux in monocytes as a response to MMK-1, whereas CHIPS does not. (42)

Subsequently, the effect of FLIPr on chemotaxis towards FPRL1 ligands was investigated by Prat et al, using Transwell systems (Fig. 25A). As expected, FLIPr inhibits chemotaxis induced by MMK-1. Only a slight inhibition of chemotaxis was shown when fMLP was used as a chemoattractant and FLIPr did not show any inhibition in chemotaxis towards C5a. Furthermore, fluorescently labeled FLIPr was added to leukocytes to investigate if FLIPr would inhibit binding of FPRL1 to its antagonists by binding to the receptor or by binding to its ligands (Fig. 25B). A significant increase in fluorescence was demonstrated for monocytes and neutrophils. Further studies showed that FLIPr also bound to NK-cells and B-cells (data not shown). (42)

To confirm that FLIPr did bind to FPRL1, Human Embryonic Kidney 293 cells (HEK293) expressing FPR, FPRL1 or C5a were constructed. Using fluorescently labeled FLIPr it was demonstrated that FLIPr bound to FPRL1, but not to FPR and C5aR (Fig. 25C). As a control, fluorescently labeled CHIPS was demonstrated to bind to the HEK293 cells transfected with FPR and C5aR. FLIPr did not show any binding to these cells, demonstrating that FLIPr specifically binds to FPRL1 in vitro. (42)

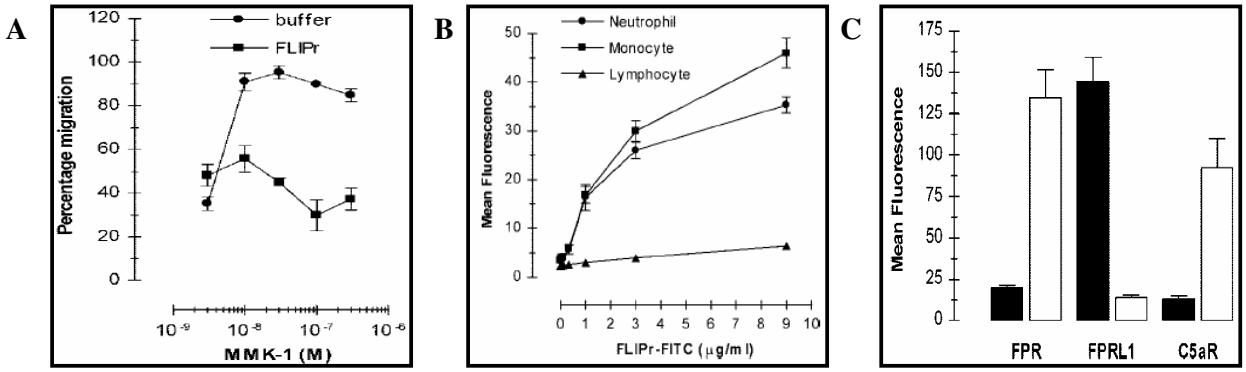

FIGURE 25. FPRL1 Inhibitory Protein binds to neutrophils and monocytes and inhibits chemotaxis of neutrophils to MMK-1. **A)** FLIPr inhibits chemotaxis of neutrophils to a FPRL1 agonist: MMK-1. Using Transwell membranes and labeled neutrophils, the effect of FLIPr on chemotaxis was measured. Cells were loaded with 3 µg/mL FLIPr or with buffer. Fluorescence of 10.000 neutrophils was measured after allowing migration for 30 minutes at 37° C. The presence of FLIPr significantly and drastically causes a decrease in neutrophil migration towards MMK-1. (42) **B)** FLIPr binding to neutrophils, monocytes and lymphocytes. At 37° C under constant shaking, cells were incubated for 30 minutes with labeled FLIPr. Cells were washed and fluorescence was measured. Data shows that FLIPr binds to neutrophils and monocytes and slightly binds to the lymphocyte population. (42) **C)** FLIPr binds to HEK293 cells tranfected with FPRL1. HEK293 cells were transfected with a vector containing FPR, FPRL1 or C5aR and labeled FLIPr or CHIPS was added to these cells (3 µg/mL). Fluorescence of the cells was measured to determine the binding of FLIPr or CHIPS. As expected, CHIPS binds to HEK293 cells transfected with FPR or C5aR, whereas FLIPr does not. FLIPr on the other hand, binds to HEK293 cells transfected with FPRL1, whereas CHIPS does not. This shows that FLIPr is binds to human cells when they express FPRL1 in vitro. (42)

In summary, FLIPr inhibits neutrophil and monocyte calcium-influx and chemotaxis towards FPRL1 ligands by binding to the FPRL1 receptor. Further studies also showed that chemoattraction by amyloid-beta, which plays a role in diseases like Alzheimer, was also abolished by presence of FLIPr. Like in many other proteins that interact with the human immune system expressed by *S. aureus*, there could one day be a key role for FLIPr in modern medicine to help fight auto-immune diseases. (42)

Chapter 4 Discussion

In this thesis, staphylococcal proteins affecting the acute inflammatory response were investigated. To better understand the interactions between *S. aureus* and the host immune system, the main components of the host immune system that are involved in fending off an *S. aureus* infection were described, prior to providing an overview of staphylococcal proteins that affect these components.

The complement system is an incredibly vast, complicated, regulated and well organized system. It is always abundantly present and through a series of possible cascades it is able to effectively kill and opsonize invading pathogens. These cascades are initiated as a result of immunoglobulins bound to the surface of the pathogen, carbohydrate ligands on the surface of a pathogen or by spontaneous hydrolysis of C3b in the presence of a pathogen. Each of these 3 pathways results in the formation of a C3 convertase, which cleave C3 in to C3a and C3b. The production of C3b results in the production of more C3 convertases as well as C5 convertases, which cleave C5 in to C5a and C5b. Binding of C5b to the surface of the pathogen results in the formation of the MAC. On top of that C3a and C5a function as chemoattractants for macrophages and neutrophils and C3b bound to the surface of the pathogen is recognized by their CR1 receptor and thus opsonizes the pathogen. (9, 10, 11)

Since this system is so complicated and has multiple points of initiation, inhibiting all pathways of the complement is the only effective way to stop complement activation. For instance: only inhibiting the lectin pathway in the complement cascade would be useless if the classical and alternative pathway would still remain intact. On that same token it would be useless to only inhibit the formation of MACs whilst ignoring the chemotaxis of neutrophils, or vice versa. It is therefore not surprising that *S. aureus* indeed is capable of inhibiting ALL parts of the complement cascade: the initiation of all 3 pathways, C3 convertases, C3, the formation of the MAC and the release of chemoattractants. (10, 12, 18, 20, 26, 28, 31, 35, 40, 41)

Gram positive bacteria like *S. aureus* have a thick peptidoglycan layer, which makes them more resistant to the MAC. The role of neutrophil recruitment is therefore very important for mounting an effective acute response against an *S. aureus* infection. (12) Neutrophils find their way to a site of infection through a process called extravasation. In capillaries they roll alongside the endothelium. When chemokines are present as a response of an infection, the neutrophils will firmly adhere to the endothelium and will then transmigrate through the extracellular matrix between the endothelial cells. Interactions between integrins and their ligands are key players in these processes. The neutrophils will then migrate through the tissue towards the site of infection by following the increasing gradient of chemoattractants like cytokines or products of the complement cascade. (12, 13, 14) Once they arrive at the site of infection, they can fend off pathogens through phagocytosis. For this to occur, neutrophils need to recognize the pathogen first. (15, 16) Neutrophils are able to recognize immunoglobulins or C3b on the surface of the pathogen, as well as formylated peptides. This will result in activation of the neutrophils, which is followed by phagocytosis of the pathogen. As can be said for the complement system, neutrophil recruitment and activation is a complex and well regulated process which can be initiated by multiple ligands. Once again, *S. aureus* is capable of inhibiting neutrophil recruitment on every level: extravasation by inhibiting rolling and tethering alongside the endothelium, chemotaxis towards cytokines and components of the complement system and activation by formylated peptides, immunoglobulins or components of the complement system. (34, 37, 39, 40. 41, 42) Some data even suggests that *S. aureus* is able to hide from neutrophils by expressing proteins that increase internalization in to host epithelial cells. (38) Since it is necessary to inhibit so many processes to effectively evade neutrophil recruitment and activation it is not surprising that *S. aureus* is capable of doing all this. However, for one pathogen to be able to intervene with this process on so many levels, as well as being able to counteract all parts of the complement system, is nothing less then impressive.

When investigating the staphylococcal proteins involved in evading the aforementioned components of our immune system it was not only the sheer number of proteins that was impressive. Remarkably some discussed proteins did not only affect different but specific proteins of the acute immune response, but they often also displayed more than one function in modulating host responses as well. Staphylokinase for instance does not only cleave IgG, but also cleaves C3b on the bacterial membrane and on top of that also inhibits bacterial killing by Human Neutrophil Protein 1 and 2. (24, 26) Thus SAK is not only a very potent opsonin degrader, but seems to be able to counter certain defensins as well. Staphylococcal super antigen-like 7 does not only inhibit the formation of MACs by binding to C5b, but also inhibits the interaction between IgA and its receptor, FcαRI. (35, 36) So not only is SSL7 able to inhibit the killing of bacteria by human serum by interacting with the complement system, data also suggests that SSL7 inhibits leukocyte activation by IgA opsonization. Most versatile of all proteins discussed was SSL5, which inhibited binding of CXC chemokines, C3a and C5a and blocks interactions with PSGL-1. (34, 41) Thus, SSL5 inhibits chemotaxis and activation induced by a number of important cytokines and by components of the complement system and on top of that inhibits the rolling and tethering of neutrophils, and by doing so inhibits both extravasation and transmigration towards the site of infection.

Remarkably, *S. aureus* has more tricks up its sleeve to evade the human immune system, besides the vast array of proteins that have already been discussed. Staphylococcal Superantigen Enterotoxin (SEA) for instance besides affecting CC chemokines receptor expression on monocytes also causes an unspecific T cell response in approximately 20% of the host T cells. (43) By doing so *S. aureus* undermines the strongest trait of the adaptive immune system: a strong response against specific antigens. Impressively, *S. aureus* is known to express not one but several different superantigens which are the causative agents for Toxic Shock Syndrome (see introduction) and food poisoning alike. (33, 34, 43) Another example is the use of leukotoxins by *S. aureus*; Alpha Toxin and Panton-Valentine Leukocidine are proteins that causes the formation of pores on host cells. These proteins are very effective in the killing of neutrophils. So not only does *S. aureus* efficiently evade the host immune system, it is actually able to target and fight it as well. (14, 44) Another remarkable strategy employed by *S. aureus* is to, if all else fails, express proteins like Staphyloxanthin or Thioredoxin that increase the chance of surviving phagocytosis by neutrophils. (14) All in all, *S. aureus* employs much more strategies and expresses even more proteins than have been discussed in this thesis.

When taking all of this in to consideration, the following questions comes to mind: Why are there so many proteins in the *S. aureus* genome that are able to counter its host immune system? And how did this come to be? One of the probable reasons why *S. aureus* acquired so many proteins that inhibit our immune responses is that *S. aureus* has a trait that is quite rare among most pathogens: besides the ability to infect the human body when immunocompromised, *S. aureus* is also a naturally occurring skin commencal (3). It has been colonizing us for a long time and in order maintain its niche it must have been vital for *S. aureus* to avoid confrontations with our immune system. Therefore, when studying *S. aureus* one must not forget that it is not a pathogen by nature, but a commencal as well. This has given *S. aureus* the opportunity to live close to our immune system and only sporadically come into contact with it. From an evolutionary point of view these are the perfect circumstances to develop proteins and traits that elude or even counter components of our immune system. And when the complexity of that immune system is taken in to consideration as well it is not surprising that *S. aureus* acquired such a high number of proteins that counter our immune system; each of these proteins gives *S. aureus* an evolutionary advantage.

So how did this come to be? Where did the genes for these proteins come from? What is often seen is that most of these proteins show large homologous regions to other proteins that are not involved in host immune evasion like proteins that allow colonization by binding to extracellular matrix compounds (in example: ClfA, Efb and Eap). It has not yet been shown that these immunomodulating proteins specifically evolved from proteins that interacted with the extracellular matrix from the host, but the genetic homology to these proteins as well as the ability of these proteins to interact with components of the extracellular matrix next to their immunomodulation functions seem to support this theory. Since these proteins were already involved in colonization of the host tissue it is not hard to imagine that specifically *S. aureus* strains that carried these genes were forced to evolve proteins to elude the hosts immune system. All in all, these proteins seem plausible progenitors for some of the proteins discussed. In certain cases it is also seen that some of the discussed proteins are highly homologous to one other (in example: CHIPS, SSL5 and SSL7). (14, 34, 39, 41) Like in the previous example it seems likely that specifically the strains carrying these proteins were subjected to evolutionary pressure to evade immune responses since they were apparently already encountering the immune system. So once again it seems like the presence of proteins that are involved in colonization and evading the human immune system perpetuates the evolution of additional immunomodulating proteins.

So how would these genes spread among different populations of *S. aureus*? A key factor in this phenomenon is the fact that very large parts of the *S. aureus* genome are in fact mobile (plasmids and phages). (4, 33) It is therefore not hard to imagine that once these genes develop and give a rise in fitness they spread among strains fast. In fact, one could also hypothesize that there may be an evolutionary advantage for the phages that carry these genes as well: viruses or phages that give their host an advantage in fitness might see an increase of their host numbers as well, indirectly resulting in larger progeny for the phages and thus increasing their fitness. For this spreading however, these strains would need to come in close contact at high frequency. Ironically, it was not *S. aureus* that has found a way to overcome this issue, ironically it was us: In hospitals there is a large number of immunocompromised people within a relatively small area. In there patients *S. aureus* has the ability to grow and undergo mutations with relatively little resistance from the immune system; there is room for trial and error. At the same time healthy people who are carrying *S. aureus* strains as a skin commencal are walking in and out of hospitals introducing and exporting new strains of *S. aureus*. While this is all happening *S. aureus* is reproducing, exchanging genes and adapting. (45) Thus, we are offering *S. aureus* an environment where there is constant alternation in immunocompromised and healthy hosts, all in the concentrated presence of different strains of *S. aureus*. All in all an unnaturally perfect environment for a pathogen to acquire traits to elude our immune system. When taking all of the aforementioned arguments in to consideration it is not just unsurprising that *S. aureus* has acquired so many proteins in its genome to counter our immune system, it would seem nothing less then inevitable.

Fortunately not all is bad news. Many of the proteins that are discovered in *S. aureus* that are utilized to evade our immune system can one day become valuable tools in the fight against auto-immune diseases. In certain diseases were monocytes or neutrophils are fighting our own organs, skin or joints perhaps we could one day be injected locally with synthetic variants of the proteins employed by *S. aureus* to inhibit recruitment of monocytes or neutrophils. Of course many of these proteins could also be used in laboratories aiding scientists in their research. A good example of one of these proteins already being put in to use is Staphylococcal protein A, which is widely utilized for the detection or removing of immunoglobulins. (30, 31)
All in all *S. aureus* is a remarkable organism that over time has developed very effective methods to evade or disable specific fragments of mammalian immune systems at a magnitude that is nothing less then impressive. Unsurprisingly, a high number of the responsible genes are even human specific (14). Although *S. aureus* is a considerable threat for immunocompromised patients, maybe we can one day benefit from the proteins that came to be as a result of the host-pathogen interactions between us and *S. aureus*. Not only in the field of science, but in the field of medicine as well.

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