Isolation and functional analysis of phage display-derived scFv-antibodies targeting the *S. aureus* SSLs

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Master's Thesis in Cell Biology

CB00BR56

Faculty of Science and Engineering

Åbo Akademi University

2020

Abstract

Staphylococcus aureus is one of the leading causes of antibiotic resistant infections in the world today. In addition to its remarkable ability to acquire resistance to antibiotics, it is also successful at suppressing the host immune defense. *S. aureus* secretes an arsenal of virulence factors that in various ways help the bacteria escape the immune system. Staphylococcal superantigen-like protein 1 (SSL1), SSL5 and SSL10 are three proteins that the bacteria produces for this purpose. In this thesis, our goal was to generate single-chain variable fragments (scFvs) that bind and inhibit the immune evasion functions of these three SSLs. Using the phage display technique, we produced different scFvs that showed binding to SSL1, SSL5 and SSL10 in an ELISA screening assay. In functional analyses, the SSL1-binding scFv-93 showed the most promise. Previous research has revealed that SSL1 inhibits several matrix metalloproteinases (MMPs). ScFv-93 was able to recover over 90% of SSL1-inhibited MMP9 activity in a fluorogenic peptide MMP activity assay. Our results indicate that by binding to SSL1, scFv-93 is able to prevent SSL1 from inhibiting MMP9. These findings might in the future help understand the mechanisms of *S. aureus* immune evasion and be a starting point for developing new therapeutics for *S. aureus* infections.

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List of abbreviations

ALP	alkaline phosphatase
C1q	complement component 1q
CDR	hypervariable complementarity-determining regions
cfu	colony forming unit
Dpa	N-3-(2,4-dinitrophenyl)-L-2,3-diamino propionyl
E. coli	*Escherichia coli*
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ENA-78	epithelial-derived neutrophil-activating peptide 78
Eu	europium
FcγR	Fcγ receptor
FDA	Food and Drug Administration
GCP-2	granulocyte chemotactic protein 2
HUVEC	human umbilical vein endothelial cells
IgG	immunoglobulin
IL	interleukin 8
IPTG	isopropyl β-d-1-thiogalactopyranoside
LA	Lysogeny agar
Mca	methoxycoumarin-4-yl-acetyl
MMP	matrix metalloproteinase
MOI	multiplicity of infection
MRSA	methicillin-resistant *S. aureus*
NET	neutrophil extracellular trap
NHS	N-hydroxysuccinimide ester
OD600	optical density of the bacteria at 600 nm
PBP	penicillin binding protein
pfu	plaque forming unit
PMN	polymorphonuclear leukocyte
pNPP	p-nitrophenyl phosphate substrate
PSGL-1	P-selectin glycoprotein ligand-1
SAg	superantigen

SB	Super Broth
scFvs	single-chain variable fragments
SSL	Staphylococcal superantigen-like protein 1
S. aureus	*Staphylococcus aureus*
TBS	tris-buffered saline
VEGF	vascular endothelial growth factor

1 Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common pathogens that causes infections, resulting in severe diseases and the treatment of the infection relies on antibiotics (Lakhundi et al., 2018). Unfortunately, the bacteria also have an extraordinary capacity to become resistant to antibiotics (Foster, 2017). This makes the treatment a long and a challenging process, with patients often having to endure long intravenous antibiotic treatments in order to get rid of the bacteria (David et al., 2019). Because of this the research into new therapeutics against the bacteria is of high priority. In addition to its ability to become resistant to antibiotics, *S. aureus* has also developed other mechanisms that it uses to promote its success as a virulent pathogen. The bacteria produce over 35 different proteins, virulence factors, that in various ways help the bacteria escape the defense mechanisms of the host's immune system (Koymans et al., 2017). One group of immune evasion proteins consists of 11 different structurally related staphylococcal superantigen-like proteins (SSLs). They all exhibit different, often several, functions that interfere with the host defense system. The proteins SSL1, SSL5 and SSL10 all have a role in helping *S. aureus* escape the innate immune system. The SSL1 and SSL5 proteins both inhibit matrix metalloproteinase activity and SSL5 additionally prevents the interaction between P-selectin and P-selectin glycoprotein ligand-1 (PSGL-1) (Koymans et al., 2016; Bestebroer et al., 2014). These functions interfere with neutrophil rolling through the vascular system and neutrophil transmigration from the vasculature into the tissue where these cells combat infections (Kolaczkowska et al., 2013). SSL10 acts by binding immunoglobulin type G1 (IgG1), which keeps neutrophils from recognizing and consequently phagocytosing pathogens (Patel et al., 2010).

In addition to using antibiotics to treat *S. aureus* infections, the immune evasion proteins could be targeted in order to neutralize them. This would give the immune system a better chance to work at its full capacity and clear out the bacteria. Previously, some *S. aureus* virulence factors have been targeted with monoclonal antibodies. For example, a monoclonal antibody called tefibazumab was developed to bind the *S. aureus* Clumping factor A in order to prevent bacterial adherence, therefore protecting against severe infections (Domanski et al, 2005). The aim of this thesis work was to use the phage display technique to produce single-chain variable fragment-antibodies

(scFvs) that bind the *S. aureus* proteins SSL1, SSL5 and SSL10 and that could possibly inhibit some of their immune evasive functions.

2 Literature review

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive bacterium that is commonly found in humans (Wertheim et al., 2005). The bacteria colonize the skin and mucosal areas of the body, but the most predominant site where the bacteria can be found is the exterior nares of the nose (Wertheim et al., 2005). It has been estimated that around 20% of the human population is persistent nasal carriers and approximately 30% is intermittent nasal carriers of the bacteria (Wertheim et al., 2005). In most cases *S. aureus* acts as a commensal, but it is still not classified as being a part of the human normal flora because it can become a pathogen and in worst cases be deadly. *S. aureus* produces a repertoire of toxins and virulence factors, which can cause different toxin-mediated diseases, such as toxic shock syndrome and staphylococcal foodborne diseases (Lakhundi et al., 2018). Infections caused by *S. aureus* are often treated with β-lactam antibiotics, however the bacteria have shown a strong capability to develop antibiotic resistance to virtually any antibiotic it is exposed to (David et al., 2019; Foster, 2017).

2.1.1 Antibiotic resistance

The first antibiotic compound, penicillin, was discovered by Alexander Fleming in 1928 when he was working with *S. aureus* bacterial cultures (Gaynes, 2017). However, it took over a decade before the drug could be purified and produced for clinical use in 1942 (Gaynes, 2017). Penicillin contains a β-lactam ring and works by interacting with the penicillin binding proteins (PBPs) that are produced by the bacteria and exhibit transpeptidase activity (Gaynes, 2017). The transpeptidation reaction is the last reaction needed when the bacterial cell wall is assembled by peptidoglycan polymerization (Reed et al., 2011). This reaction is prevented by penicillin (Gaynes, 2017).

Only a few years after penicillin was introduced for clinical use, the first cases of penicillin resistant *S. aureus* were reported (Chambers et al., 2009). Penicillin resistance is based on the bacteria producing a penicillinase enzyme that hydrolyzes the β-lactam ring of penicillin that accounts for its antimicrobial activity (Chambers et

al., 2009). Later, methicillin, resistant to hydrolysis by penicillinase, was introduced as a new type of antibiotic for treating penicillin-resistant *S. aureus* infections, but again shortly thereafter new strains of the bacteria were found to be resistant to the treatment (Chambers et al., 2009). The first strains of methicillin-resistant *S. aureus* (MRSA) had been found. MRSA is generated when a bacterium receives the methicillin resistance gene, *mecA* that is carried by a mobile genetic element, staphylococcal cassette chromosome *mec* (Lim et al., 2002). The *mecA* gene encodes for PBP2a that is a different type of PBP protein than what *S. aureus* normally produces and has a lower affinity for β-lactams such as penicillin and methicillin (Lim et al., 2002). This allows the PBP2a protein to stabilize the bacterial cell wall with its transpeptidase activity. The origin of this gene is unknown, but it is speculated that it can be transmissible between different staphylococcal species (Tsubakishita et al., 2010).

Initially, MRSA was considered only a healthcare-associated disease as it was mostly encountered in hospital settings, but in recent years community-associated spreading has been a growing problem as well (Lakhundi et al., 2018). Today MRSA is one of the main causes of antibiotic resistant infections in Europe and therefore it is a large burden on the healthcare system. In 2015 MRSA gave rise to approximately 150 000 infections out of which 7000 were deadly (Cassini et al., 2019). Because of this WHO has classified the development of therapeutics against antibiotic resistant strains of *S. aureus* as a high priority (WHO, 2017).

2.2 Neutrophils have an essential role in bacterial elimination

Neutrophils are the primary immune cells that are involved in a rapid innate defense for combating bacterial infection, before the immune cells that are a part of the acquired immune system can take over (Malech et al., 2014). They are produced in large numbers (up to $2x10^{11}$ cells per day) in the bone marrow from myeloid precursor cells and are the most abundant polymorphonuclear leukocytes (PMN) in the blood (Borregaard, 2010). PMNs are a group of white blood cells that are characterized by their cytoplasm being enriched with secretory granules and by a segmented nucleus (Breedveld et al., 2017). Neutrophils use three different mechanisms for killing bacteria: phagocytosis, release of antibacterial proteins or production of neutrophil

extracellular traps (NETs) (Kolaczkowska et al., 2013). However, before neutrophils can start the killing of bacteria, they have to be attracted and migrate to the site of infection. *S. aureus* has developed ways to target all these different neutrophil functions in order to prevent the bacterial elimination.

2.2.1 Neutrophil movement through the vascular system

The number of neutrophils increases during inflammation and they are recruited to the site of infection by inflammatory mediators (such as histamine and cytokines) that are released by leukocytes when they come in contact with pathogens (Kolaczkowska et al., 2013). Neutrophil migration can also be stimulated by the increased amount of P-selectin on endothelial cell surfaces. The endothelial cells are activated by pattern-recognition receptors that stimulate the secretion of P-selectin from Weibel-Palade bodies (Bestebroer et al., 2011). Neutrophil movement is facilitated by a process called neutrophil rolling that is carried out by PSGL-1, expressed on the neutrophil surface, interacting with P-selectin on the endothelium of blood vessels (Figure 1) (Bestebroer et al., 2014). When neutrophils reach the site of infection they need to leave the vasculature, first crossing the endothelium and then the basement membrane (Ley et al, 2007).

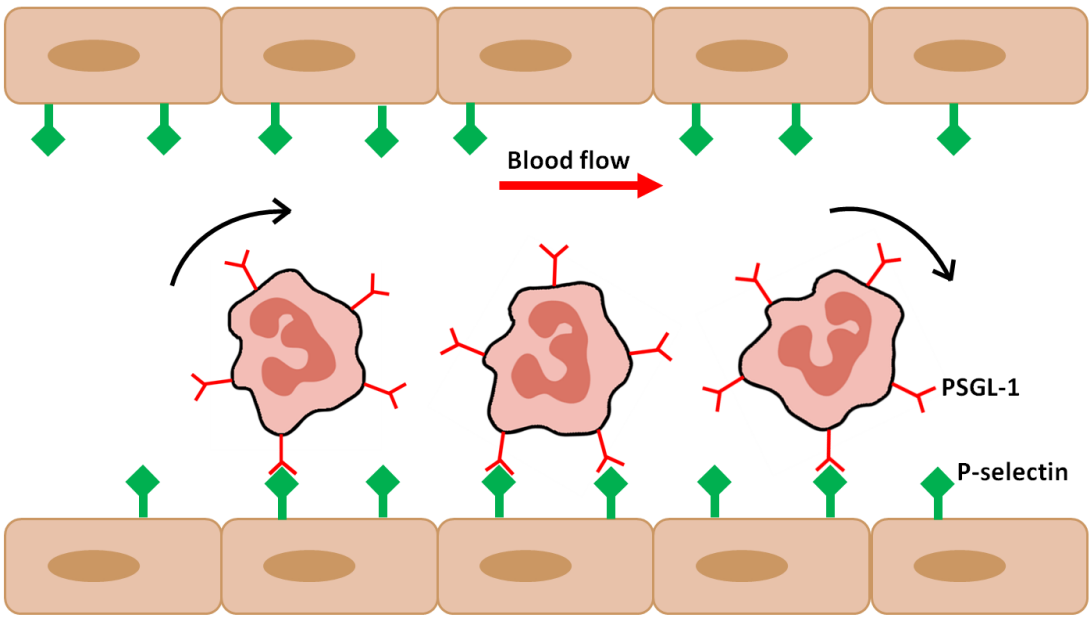

Figure 1. Neutrophil movement through the vascular system. The neutrophil rolling is mediated through the binding of PSGL-1, located on the neutrophil surface, and P-selectin on the surface of endothelial cells (modified from McEver et al., 2010).

2.2.2 Phagocytosis

Once the neutrophils encounter the pathogens, they can start eliminating them by phagocytosis. In order for a neutrophil to recognize a bacterium as a pathogen it needs to eliminate, the bacterium has to be opsonized. IgG and complement component 3b function as opsonins that have attached to the pathogen surface (Lee et al., 2003). The neutrophil binds to these opsonins with Fc receptors and a subgroup of β2 integrins (Lee et al., 2003). The cell membrane then stretches around the bacteria until it is completely entrapped inside a vacuole where the pathogen is broken down by proteolytic enzymes, antimicrobial proteins and reactive oxygen species (Brinkmann et al., 2004).

2.2.3 Neutrophil extracellular traps

Another mechanism neutrophils use to combat pathogens, such as bacteria, is by producing NETs. These intracellular structures that are released into the extracellular environment during inflammatory conditions are largely composed of DNA, in addition to histones and granular proteins (Kolaczkowska et al., 2013). The obvious way that NETs can interfere with bacterial infections is by capturing the bacteria in the web-like formations and preventing the spread from the initial site of infection (Brinkmann et al., 2004). However, the structures have also been shown to function by degrading virulence and by killing the bacteria, again limiting the bacterial infection (Brinkmann et al., 2004). In addition to neutrophils using this defense mechanism against *S. aureus*, it has also been shown that other PMNs and macrophages can form extracellular traps as a response against *S. aureus* (Chow et al., 2010).

2.2.4 The role of matrix metalloproteinases in neutrophil recruitment

Matrix metalloproteinases (MMPs) comprise a family of calcium-dependent zinc-containing proteinases (Verma et al., 2007). These enzymes were first described as proteinases that break down extracellular matrix (ECM) components, but later they were found to contribute in a number of cellular processes, one of them being the immune defense (Koymans et al., 2016). The ability to degrade ECM is needed for effective immune cell migration to infected areas by removing physical barriers.

However, the MMPs have been shown to facilitate immune cell recruitment through several other mechanisms (Stefanidakis et al., 2004; Lin et al., 2008). A wide range of MMPs are involved in the release of ECM-bound pro-inflammatory signals or in the potentiation of chemoattractant molecules (Koymans et al., 2016).

Neutrophils secrete two MMPs; collagenase (MMP8) and gelatinase B (MMP9), whose function in the immune system will be discussed in more detail. MMPs are secreted in a proenzymatic form and are activated extracellularly (Verma et al., 2007). Both MMP8 and 9 can break down collagen types I-III, that are the most abundant types of protein in the ECM (Van Doren, 2015). They also have important functions in activational cleavage of chemokines. Both proteases are able to modulate the granulocyte chemotactic protein 2 (GCP-2) cytokines activity and MMP9 is additionally able to activate epithelial-derived neutrophil-activating peptide 78 (ENA-78), interleukin 8 (IL-8) and IL-1β (Van den Steen et al., 2003; Schönbeck et al., 1998). Neutrophil secretion of MMP8 and 9 is stimulated by IL-8 and the release in turn enables MMP9 processing of IL-8 into a 10-30-fold more active chemokine, generating a positive feedback loop (Van den Steen et al., 2000). The cytokines GCP-2, ENA-78 and IL8 are all involved in neutrophil recruitment whereas IL-1β has been linked to NET formation, in addition to their other functions in the immune system (Disteldorf et al., 2014; Proost et al., 1993; Harada et al., 1994; Meher et al., 2018).

2.3 *S. aureus* immune evasion

One important reason to why *S. aureus* is so successful as a pathogen is its ability to avoid the host's immune system. To date, there are 35 different known virulence factors that *S. aureus* uses for avoiding the immune system (Koymans et al., 2017). This is the highest number of immune evasion proteins recorded in any other bacteria. The innate immune system that is the first line of defense against bacterial infections, is the primary target for these proteins. The immune evasion proteins affect the effectiveness of neutrophils and macrophages, the activation of the complement system and help bacteria escape phagocytosis. *S. aureus* also secretes a few virulence factors that can disturb the adaptive immune system by interfering with the production of a functional immunological memory.

2.3.1 Mechanisms of immune evasion

S. aureus immune evasion proteins can be divided into four categories based on their function (Koymans et al., 2017). The main mechanism the proteins use is to interfere with ligand-receptor interactions or to occupy the catalytic sites of enzymes in order to evade the immune system (Bestebroer et al., 2007). For example, the SSL1 and SSL5 proteins inhibit MMP8 and 9 activity, which interferes with neutrophil migration (Koymans et al., 2016). By blocking the mechanisms that are used for bacteria recognition and immune cell recruitment, the inflow of immune cells to the infection site is limited (Koymans et al., 2017).

The second strategy *S. aureus* uses is the production of enzymes that cleave important molecules in the immune system. Components of the complementary system and antibodies are cleaved in order to reduce opsonization, and cell surface receptors are cleaved to interfere with immune cell signaling (Koch et al., 2012; Laarman et al., 2012). One example is the staphylococcal enzyme staphylokinase that breaks down IgGs that are important opsonins (Rooijakkers et al., 2005).

The third approach the bacteria uses is the production of toxins that result in the destruction of immune cells (Nygaard et al., 2012). *S. aureus* releases α-hemolysines that generates pore formations on the surface of the host cell membranes which lyse the cells. Especially monocytes, B cells, and T cells are sensitive to α-hemolysines, whereas neutrophils are less affected.

The last strategy entails the bacteria activating the immune system in a way that favors the bacterial survival. As an example, staphylococcal superantigens (SAgs) that bind T cell receptors and major histocompatibility complex class II cause a massive nonspecific activation of T-cells that is several times larger than a normal immune response (Koymans et al., 2017). SAgs are the strongest known T cell mitogens, as less than 0.1 pg/ml of bacterial antigen can stimulate T cells into uncontrollable proliferation (Proft et al., 2003). It is not entirely clear how the bacteria benefits from such a reaction, but it can be speculated that the recruitment of T cells could suppress the activation of neutrophils and macrophages that are a bigger concern for the bacteria (Fraser et al., 2011).

2.3.2 Superantigen-like proteins

In this project we have chosen to focus on three of the *S. aureus* immune evasion proteins: SSL1, SSL5 and SSL10. Together with 11 other structurally similar proteins they comprise the superantigen-like protein group (McCarthy et al., 2013). These proteins have acquired their name because they structurally resemble the superantigen proteins such as toxic shock syndrome toxin-1. However, they lack the T cell activating properties (Fraser et al., 2008). The SSL1-11 are encoded in the genomic island α and the SSL proteins 12-14 are encoded in the immune evasion cluster 2 (McCarthy et al., 2013). The expression of these proteins varies between different *S. aureus* strains (Oku et al., 2018). The SSLs have been shown to exhibit immune modulating properties. However, the function of some of them has not yet been discovered and there are most likely other functions yet to be assigned to the SSL proteins that exhibit already known activities.

2.3.2.1 SSL1 and SSL5

The SSL5 protein has been shown to be involved in several processes that inhibit the host innate immune system. Firstly, the protein binds to PSGL-1 and simultaneously prevents the ligand from binding to P-selectin (Bestebroer et al., 2014). As mentioned earlier, P-selectin is expressed on activated endothelial cells whereas PSGL-1 is expressed on neutrophils, but also on most of the other leukocytes such as monocytes and T cells. Bestebroer et al. (2014) also investigated the SSL5 proteins effect on neutrophil rolling in a flow chamber, where a coverslip was coated with P-selectin or with human umbilical vein endothelial cells (HUVECs). The addition of SSL5 dose-dependently inhibited the attachment of neutrophils to the P-selectin coated coverslip and 73% of rolling adhesion to the HUVECs was inhibited by a 10 µg/ml concentration of SSL5.

The second way SSL5 interferes with the host's defense against invading *S. aureus* is by inhibiting several MMPs. Koymans et al. (2016) demonstrated that both SSL1 and SSL5 inhibited the enzymatic activity of all of the eight MMPs (MMP1, 2, 7, 8, 9, 12, 13 and 14) that they tested, in a concentration dependent manner. These included the neutrophil secreted MMP8 and 9.

2.3.2.2 SSL10

The amino acid sequence of SSL10 is more distantly related to the sequences of SSL1 and 5 (Supplemental figure 6). Nevertheless, SSL10 is involved in weakening the host defense mechanisms. The protein binds to IgG1 and has been reported to block the interaction between IgG1 and its targets: Fcγ receptor (FcγR) and complement component 1q (C1q) (Itoh et al., 2009). The inhibition of C1q binding to IgG1 leads to the suppression of the complement activation through the classical pathway (Itoh et al., 2009). The C1q is the initiating factor of the complement system that consists of a group of small proteins that via a signaling cascade result in the formation of a membrane attack complex on the membrane of pathogens and damaged host cells (Xie et al., 2020). The FcγR receptor mediates numerous inflammatory mechanisms such as phagocytosis, antibody-dependent cellular cytotoxicity and activation of immune cells (Patel et al., 2010). SSL10 has been shown to be able to inhibit phagocytosis by monocytes and neutrophils by blocking the interaction between IgG1 and FcγR (Patel et al., 2010).

Unpublished results have also shown that the SSL10 protein binds to DNA (personal communication with Assistant Professor Dr. Carla de Haas, UMC Utrecht). One hypothesis for this is that the binding might influence NET-based immune clearance. *S. aureus* has previously been shown to use strategies to interfere with NETs in order to insure a more successful infection. For example, the bacteria secrete an extracellular nuclease that makes it more resilient against NET-mediated killing (Berenders et al., 2010). In addition, the *S. aureus* secreted extracellular adherence protein binds and aggregates DNA, which modulates the formation and stability of NETs. Therefore, research into the effects of the SSL10 binding to DNA becomes interesting.

2.4 Phage display

Phage display is a technique utilized for rapid selection of proteins with desired specificity and affinity for a target molecule. The advantage in this technique is the linkage of the phenotype of a protein to its genotype (Smith et al., 1997). Bacteriophages are used to express a protein of interest by cloning its gene into the phage genome or phagemid vector, as a fusion protein with one of surface proteins of the phage (Frei et al., 2016). The pIII coat protein on a M13 phage is typically used as

it is located on the tip of the phage and usually there are only five copies of them (Smith et al., 1997). A phage library is built up of a heterogeneous mix of phages displaying fusion protein clones that have been mutated into slightly different versions (Frei et al., 2016). A typical phage library contains around a billion phages (Smith et al., 1997). The selection process is done by allowing the phage library to bind to a specific target protein and the unbound phages are discarded (Figure 2). The remaining phages represent a subpopulation of phages with binding affinity to the target. The phages can then be amplified by infecting bacterial host cells that will produce millions of copies of them, producing a new phage library stock. This stock is then used for a new selection towards the target. The whole process, called a panning round, is repeated (usually for a total of two to four rounds) until a phage stock containing a target specific selection of phages is obtained (Smith et al., 1997). Then the amino acid sequence of the fusion protein presented on the phages can be determined by sequencing the corresponding gene in the viral DNA.

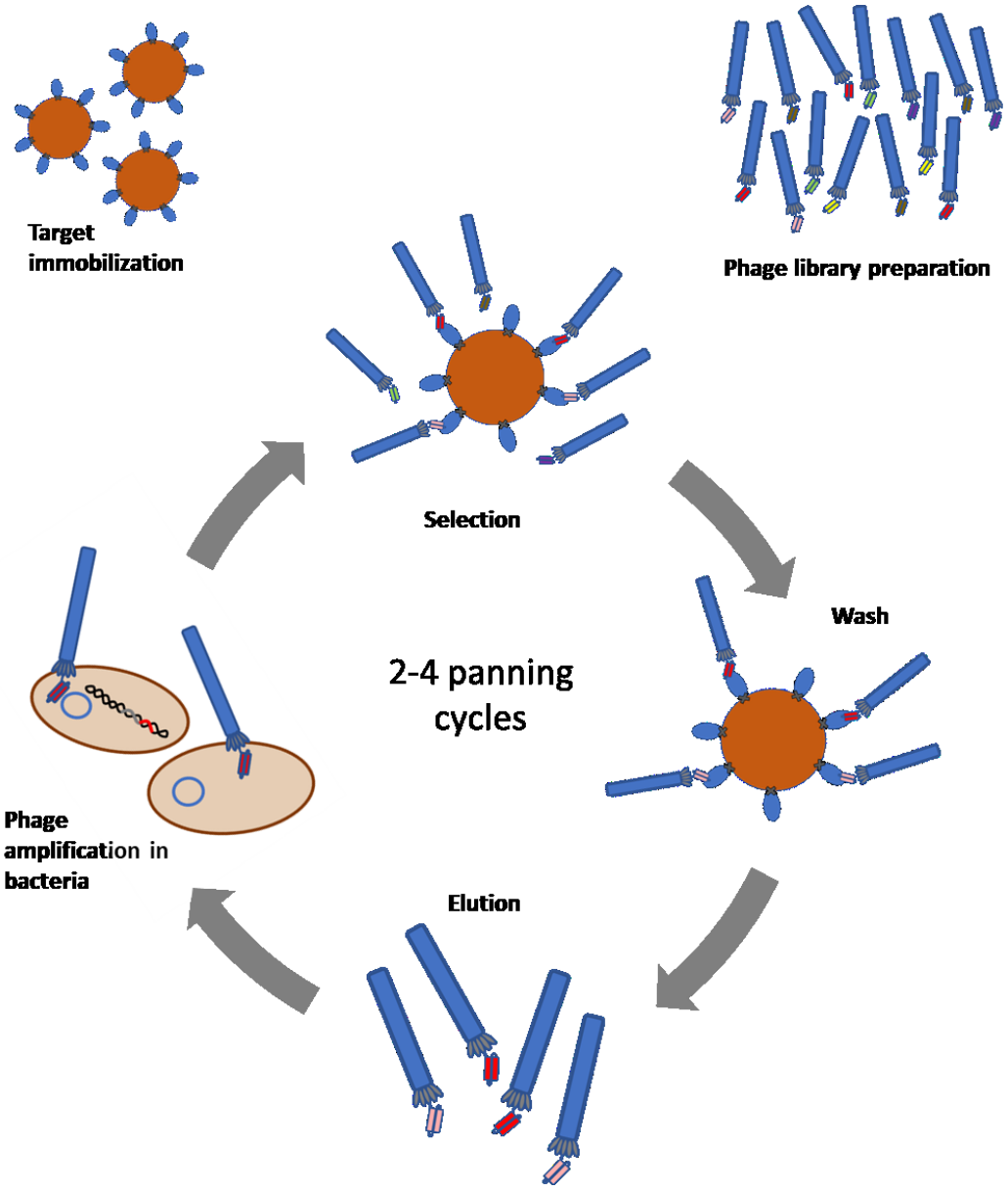

Figure 2. Flow chart of a panning cycle in a phage display experiment. A phage library encoding for slightly different versions of an scFv-fragment is combined with the target that has been immobilized to a surface (for example magnetic beads). The unbound phages are then washed away, the phages are eluted and a new phage library is propagated in bacteria. This new phage library is used for another cycle and the process is repeated several times, depending on the desired outcome.

2.4.1 Bacteriophages

Phages are viruses that infect bacteria and are often used as recombinant DNA vectors in research (Smith et al., 1997). The filamentous M13 phage has commonly been utilized for phage display. These phages infect *Escherichia coli (E. coli)* through binding of the tip of the F-pilus that *E. coli* uses for exchange of genes via conjugation (Hu et al., 2019). The F-pilus is depolarized during phage infection, which means that each bacterium, as they have only one F-pilus, can be infected by no more than one phage (Schmiz et al., 2000). The wild type phage contains a genome consisting of (+) single-stranded DNA that encodes for 11 different proteins (Ledsgaard et al., 2018). Five of them are coat proteins and the rest help with replication and assembly of the virion. The pIII coat protein that is often used as the display-protein in phage display, binds the F-pilus during the first stage of infecting *E. coli*. After the single-stranded chromosome has been inserted into the bacteria, it is converted into double-stranded DNA (Ledsgaard et al., 2018). Viral proteins encoded by the DNA are produced and the viral genome is replicated using the (-) strand as the template (Ledsgaard et al., 2018). The new phage particles are then assembled through a pore in the bacterial membrane, without lysing the bacteria (Ledsgaard et al., 2018).

2.4.2 Phagemid vectors

In phage display phagemid vectors are often used instead of complete M13 phages (Figure 3). A phagemid is a type of plasmid that carries only one of the phage structural proteins (often pIII), which is fused with the protein that is to be displayed (Frei et al., 2016). The phages that only contain a phagemid therefore lack the genes for enzymes and structural proteins required for phage progeny production. Phage display experiments using these types of phages to display proteins need to include a helper phage (Frei et al., 2016). These phages contain the rest of the genes needed for a successful replication. The helper phage genome has been mutated so that its packing ability has been reduced and the packaging of the phagemid is favored instead (Frei et al., 2016). This ensures that the progeny produced are suitable to be used for the next round of panning. The phagemid also carries two types of replication origins; it has one bacterial origin of replication for propagation in *E. coli* and one F1 phage origin for replication as single stranded DNA (Frei et al., 2016).

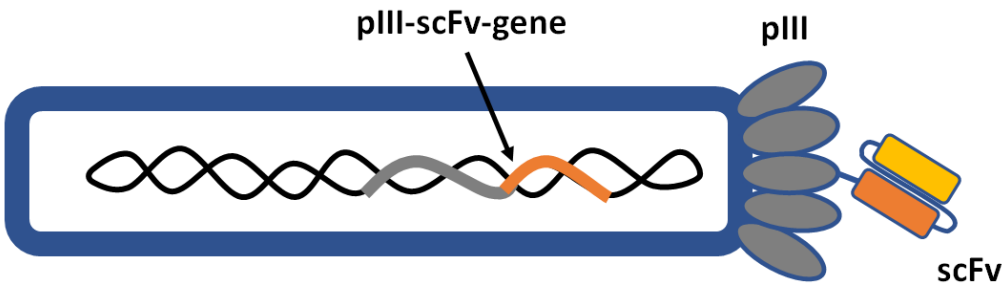

Figure 3. Schematic structure of a M13 phagemid vector. There are often five copies of the structural phage protein pIII located on the tip of the M13 phage. A scFv-gene has been fused with the gene encoding for the pIII-protein, resulting in the scFv antibody being displayed on the surface of the phage (modified from Ledsgaard et al., 2018).

2.4.3 Antibody phage libraries

Phage display has often been used for the selection of antibodies that have later been developed into therapeutics. Antibodies, also known as immunoglobulins, are produced by B lymphocytes and used in the humoral immune defense against pathogens. Out of the different types of immunoglobulins, the IgG type is the most commonly used in both therapeutics and diagnostics (Lu et al., 2020). The phage library used for isolation of binding immunoglobulins can be produced in different ways. In the first antibody producing phage display experiments the antibody phage libraries were made from immunized animals or humans that had developed a specific immune profile from having an autoimmune disease, cancer or being exposed to an antigen (Hoogenboom et al., 2000). Then it was discovered that antibody libraries derived from nonimmunized humans (naive libraries) were also sufficient at producing binders to various antigens. And later, libraries with synthetically derived antibodies were developed. All of these systems are still used because of their different pros and cons. Immune libraries have a higher proportion of binders because of the natural affinity maturation of the antibodies (Hoogenboom et al., 2000). However, it is a laborious process as a new library has to be constructed for every antigen. A naive library can be used for several antigens and the structure of the antibodies have been selected through evolution (Hoogenboom et al., 2000). The disadvantage of antibodies generated by the immune system is the bias that prevents the production of self-binding antibodies (Chen et al., 2014). The synthetic libraries are much more

customizable and are not limited by bias, but require a more considered designing (Hoogenboom et al., 2000).

The production of a synthetic antibody library requires a lot of expertise in antibody structure and function. A framework is chosen based on the knowledge we have about optimal binders and then the binding site needs to be diversified by mutation (Chen et al., 2014). An IgG is built up of two heavy (H) and two light (L) chains that together form a Y-shaped molecule (Figure 4) (Schroeder et al., 2010). A light chain consists of one variable domain and one constant domain, whereas a heavy chain has two additional constant domains. In an IgG the chains are connected with disulfide bridges so that the variable domains come into close proximity at the tips of the Y-arms forming two antigen binding sites. At the end of each variable domain there are three loops, from anti-parallel β-sheets, that are called the hypervariable complementarity-determining regions (CDR) (Schroeder et al., 2010). The diversity in these CDR-loops accounts for the strong binding ability of antibodies. In natural antibody formation the most diversity comes from varying the length and structure of the third CDR-loop on the heavy chain (CDR-H3) (Schroeder et al., 2010). Synthetic antibody phage libraries are often designed so that the CDR-H3 region is diversified the most. In the other CDR loops there are specific locations that are hot spots for hypermutation in natural antibodies, and these are also targeted for variation during the library design (Hoogenboom et al., 2000).

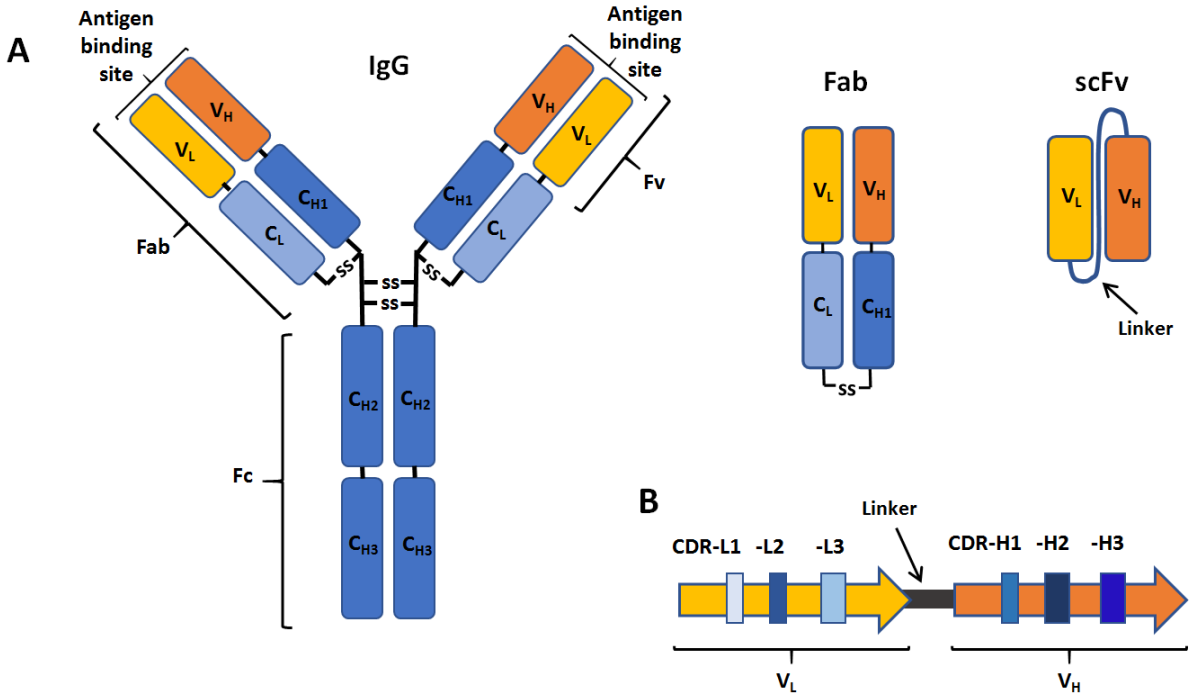

Figure 4. Schematic structure of IgG and antibody fragments. **A)** The IgG heavy chain contains one variable domain (V_H) and three constant domains (C_H) whereas the light chain only contains one variable (V_L) and one constant (C_L) domain each. The IgG can be divided into different parts such as: The Fc (crystallizable fragment) region, two Fab (antigen binding fragment) regions and two Fv (variable fragment) regions. The Fc and Fab regions are connected by disulfide bridges. The Fv part needs to be connected with a polypeptide linker in order for it to form a functional fragment on its own, creating a scFv. **B)** The structure of a scFv fragment gene. Both the V_L and the V_H contain three CDR regions (modified from Brockmann et al., 2010).

2.4.4 ScFv phage libraries

The large size of a complete antibody makes it difficult to express functional versions of them in bacteria (Hoogenboom et al., 2000). Therefore, alternative display-variants of recombinant antibodies such as scFv are often used. A scFv is smaller in size as it only consists of the variable regions of one light and one heavy chain which are linked together with a short, flexible polypeptide linker (Ahmad et al., 2012). This makes up the unit of an antibody that still exhibits the antigen binding capacity (Ahmad et al., 2012). ScFvs are folded more efficiently than whole antibodies that have more complicated folding and glycosylations, which often makes them the preferred option for phage display libraries (Ahmad et al., 2012). The scFvs can be expressed into the bacterial periplasm, the space between the inner and outer cytoplasmic membrane

(Ahmad et al., 2012). A signaling peptide is often used to ensure the direct secretion of the scFv into the periplasmic space that contains proteins such as chaperones that help proteins fold correctly (Rosano et al., 2014). Even if scFv phage libraries are often used to develop new therapeutic leads, the whole antibody format has mostly been used for the final product. Once a high affinity scFv-binder has been produced with phage display, it can be converted into an IgG or a Fab format (Steinwand et al., 2014).

2.5 Antibodies as therapeutics

In 1986, the first monoclonal antibody was approved for therapeutic use by the US Food and Drug Administration (FDA) and since then the antibody engineering technology has improved greatly (Lu et al., 2020). The number of antibody drugs on the market has drastically increased over the years and in recent years they have been the leading class of new drugs developed (Lu et al., 2020). The major advantage of antibody drugs is their high specificity that results in fewer adverse effects and their long elimination half-life requiring fewer doses for a therapeutic effect (Keizer et al., 2010).

Majority of the antibodies that have been approved for therapeutic use are in an IgG and a few in a Fab format (Lu et al., 2020). However, research into using scFvs in therapeutics is increasing as they have properties that can be favorable in certain therapeutic approaches. Compared to whole antibodies, scFvs are effectively able to penetrate tissue and circulate more rapidly in the blood and can therefore be localized quicker to their site of action (Pucca et al., 2011). They are also cleared from the blood more rapidly and have a lower retention in the kidneys (Ahmad et al., 2012). These traits can for example be useful in cancer as they could quickly penetrate and localize into tumors and their effective clearance makes them safer if coupled to a molecule that is harmful to the healthy tissue (Ahmad et al., 2012). Recently, the first humanized scFv, Brolucizumab, was approved by the US FDA (Lu et al., 2020). It is used for the treatment of age-related macular degeneration and it binds vascular endothelial growth factor (VEGF), thus inhibiting the activation of VEGF receptors (Dugel et al., 2020).

3 Aims

S. aureus infections have been proven to be especially difficult to combat as the bacterium has an extraordinary capacity to achieve resistance against different antibiotics and it expresses a large amount of virulence factors that help it establish a stronger infection (Koymans et al., 2016; Foster, 2017). Currently, antibiotics are used to treat these infections, but drugs targeting the virulence factors could be useful to complement the existing treatments. In this thesis, the goal was to produce antibodies that recognize and inhibit virulence factors that could be further developed to be used alongside an antibiotic in the treatment of *S. aureus* infections. As our targets we chose the *S. aureus* immune evasion proteins SSL1, SSL5 and SSL10. The specific aims of this study were to:

- Use the phage display technique to select scFv-antibodies that bind specifically to SSL1, SSL5 or SSL10.
- Clone the genes for the selected scFvs into *E. coli* and produce them by periplasmic expression.
- Use an enzyme-linked immunosorbent assay (ELISA) to screen for the best binding clones to be sequenced.
- Use functional assays to determine the scFvs ability to prevent:
    - SSL1 or SSL5 from inhibiting MMP9 activity
    - SSL5 from interfering with PSGL-1 and P-selectin interaction
    - SSL10 from binding DNA

4 Methods

In this thesis we used the phage display technique to produce scFv-antibodies that bind to the *S. aureus* proteins SSL1, SSL5 or SSL10 (the antigens). The target proteins were labeled with biotin and were attached to streptavidin coated beads, which were used in three rounds of panning with two different scFv-phage libraries. After the enrichment of SSL-binding phages, the genes encoding for the scFv-fragments were cloned into Mach1-T1 bacteria as a fusion protein with alkaline phosphatase (ALP). The scFv-ALP fusion proteins were then expressed in the bacteria and could then be tested in binding assays from where the most promising binders could be chosen. These scFvs where then purified and tested in different functional assays to examine if they had any effect on the SSL proteins function.

4.1 Phage display

4.1.1 Phage library

Two different synthetic scFv phage libraries were used in this project. A scFvM2, enlarged hapten or multipurpose library and a scFvP monovalent protein library that both have been developed and produced by Urpo Lamminmäki's group (Biotechnology, Turku University) (Huovinen et al., 2013). For the first panning round the libraries were combined by mixing $2.5x10^{12}$ colony forming units (cfu) of both libraries to a total volume of 2 ml TBT-0.05. In the following panning rounds $1x10^{11}$ cfu phages derived from the previous panning round were diluted in 1 ml TBT-0.05 and was used for the next panning rounds.

4.1.2 Negative selection of phages against streptavidin

Before each selection round the phage library was subjected to a negative selection in order to avoid the enrichment of phages that bind to the streptavidin on the magnetic beads. A streptavidin coated microtiter plate (41-07TY, Kaivogen, Finland) was washed three times with wash solution (42-01, Kaivogen, Finland) with a Delphia plate washer (Perkin Elmer, USA). Then the phage solution that was to be used for a panning round was distributed on the microtiter plate. This was incubated on a plate

shaker at room temperature for one hour. Then the unbound phages were collected and the streptavidin bound phages were discarded. The phages where then blocked with 10 µM biotin and incubated in rotation at room temperature for one hour before they were ready to be used for the panning.

4.1.3 Biotin labeling of the antigens

The antigenic targets for the phage display experiments were the proteins SSL1, SSL5 and SSL10 (produced in Jos A.G. van Strijp's group, Utrecht, the Netherlands). In order to attach the proteins to streptavidin and neutravidin coated magnetic beads the proteins had to be biotinylated. The interaction between biotin and streptavidin is one of the strongest known non-covalent interactions and is often utilized in molecular biology (Meyer et al., 2006). Neutravidin is a deglycosylated version of avidin that differs from streptavidin at the sequence level but has a very similar structure and binding affinity to biotin. The SSL proteins were coupled with a five-molar excess of NHS-PEG4-Biotin by using the EZ-Link NHS-PEG4-Biotinylation Kit (Thermo Scientific, USA). The N-hydroxysuccinimide ester (NHS) reacts with the primary amino groups on proteins forming an amide bond and releasing the NHS as a byproduct of the reaction. Afterwards the buffer of the biotinylated proteins was changed to tris-buffered saline (TBS) using NAP^{TM} Columns (GE Healthcare Life Sciences, UK).

4.1.4 Immobilization of antigen on streptavidin coated beads

In phage display the target protein for the phage library needs to be immobilized on a surface to facilitate the separation of the protein-phage complex after the selection. Streptavidin coated M280 Dynabeads® (Invitrogen, USA) were prewashed three times with TBT-0.05 (chapter 11) each time using a magnet collecting the magnetic beads. For the first round of panning 50 µl of beads were used and for the following rounds the amount was lowered to 5 µl. To prevent the scFvs enrichment towards the bead coating, the beads were alternated between streptavidin and neutravidin coated M280 Dynabeads® (Invitrogen, USA) in between the panning rounds.

To saturate 5 µl beads with biotinylated antigen 0.8 µg of SSL1, 2.0 µg of SSL5 or 0.28 µg of SSL10 was used. The beads and the protein were diluted in 0.5 ml TBT-0.05 and incubated in rotation at room temperature for 30 minutes. The unbound streptavidin/neutravidin on the beads was then blocked by adding 100 µM of D-Biotin (Sigma-Aldrich, USA) and incubated at room temperature for one minute. Finally, the beads were washed three times with TBT-0.05.

4.1.5 Selection

The phage library selection was done by allowing the target protein coated beads and the scFv presenting phage library to interact. In the first panning round the selection was done by incubating at 4°C overnight and for the rest of the panning rounds it was done by incubating at room temperature for two hours. The beads and phages were incubated in rotation. For each selection a negative control, using uncoated beads, was performed. This is discussed later in chapter 4.1.8. Afterwards the beads were washed three times with TBT-0.05 and one time with TBS with the help of a magnet. After washing away the unbound phages, the phages bound to the beads could be collected by elution. The phages have been designed with a trypsin cleavage site that cuts off the scFv-fusion protein from the phage, which allows for elution by suspending the beads in 60 µg/ml trypsin (Sigma-Aldrich, USA) diluted in TBS, at room temperature for 30 minutes. The trypsin was then inhibited by adding soybean trypsin inhibitor (Sigma-Aldrich, USA) to a final concentration of 50 µg/ml.

4.1.6 Phage amplification in XL1-Blue *E. coli*

4.1.6.1 Infection

The eluted phages needed to be amplified in bacteria in order to form a new phage stock. *E. coli* (XL1-Blue) cells were inoculated from a glycerol prep to Super Broth (SB) medium (chapter 11) with 0.2% glucose and 10 µg/ml tetracycline (Sigma-Aldrich, USA), and grown at 30°C, 300 rpm, overnight. The next day a small amount of the cells were transferred to fresh culture media and grown to the logarithmic phase, at 37°C and 300 rpm, to be used for phage infection. The logarithmic growth phase

was reached when the optical density of the bacteria at a wavelength of 600 nm (OD600) was between 0.3and 0.6.

In the first panning round 5 ml of XL1-Blue cell culture was used to infect the bacteria with the eluted phages, whereas in the following panning rounds only 1 ml of cell culture was used. The bacteria and the phages were incubated at 37°C without shaking for 30 minutes. The infection was done without shaking so that the bacteria pili that the phages utilize for their entry into the bacteria, would not be broken by the force. Thereafter the cells were plated on Lysogeny agar (LA) plates (containing 10 µg/ml tetracycline and 25 µg/ml chloramphenicol (Sigma-Aldrich, USA)) and incubated at 30°C overnight. The phagemid contains a chloramphenicol resistance gene, which is used as an indicator of the infected bacteria.

4.1.6.2 Helper phage infection

The following day the cells were collected from the plates. The OD600 was measured and the bacteria were diluted to an OD600 of 0.1 in 20 ml SB medium (1% glucose, 10 µg/ml tetracycline and 25 µg/ml chloramphenicol). The bacteria were then grown at 37°C, 300 rpm, until they reached the logarithmic growth phase. The remaining cells from the overnight culture were stored at -70°C after adding approximately 15% glycerol, later to be used for plasmid DNA extraction.

Approximately 20 MOI of VCS M13 helper phage (200251, Agilent Technologies, USA), 2 µl of a $1x10^{14}$ pfu/ml stock was added and was allowed to infect at 37°C, without shaking, for 30 minutes. The multiplicity of infection (MOI) means the average number of phages that will be infecting one cell and plaque forming unit (pfu) is a measure of the number of infectious virus particles. The helper phage provides the genes that are needed for the scFv-displaying phages to replicate and a gene for kanamycin resistance. After this, the residual helper phages needed to be removed so the bacteria were cooled on ice and the cells were collected by centrifugation at 4°C, 4000 rpm for 10 minutes. The cells were then resuspended in 20 ml of glucose-free SB medium (10 µg/ml tetracycline and 25 µg/ml chloramphenicol) and incubated at 30°C, 300 rpm for one hour. After which 50 µg/ml kanamycin (Sigma-Aldrich, USA) and 100 µM isopropyl β-d-1-thiogalactopyranoside (IPTG) (Fisher Bioreagents, USA) was added and the bacteria were allowed to grow at 26°C, 300 rpm, overnight. By

growing the bacteria in glucose-free media and adding IPTG the transcription of the phagemid is induced.

4.1.6.3 Phage stock preparation

After the phages had been replicating overnight in the XL1-Blue bacteria, they were ready to be harvested. By centrifuging the bacterial culture, at 4°C, 12 000 g (Beckman Coulter Avanti J-26XP, USA) for 10 minutes, the cells could be discarded and the phage containing supernatant was obtained. Next, 1/5 of the supernatant volume of PEG/NaCl was added and the phages were precipitated on ice for at least 30 minutes. Then the phages were pelleted by centrifugation at 4°C, 10 000 g for 20 minutes. The supernatant was removed and the pellet was suspended in 1 ml TBS. To get rid of any residual cells, the solution was centrifuged at 4°C, 16 000 g for five minutes and the phages were again precipitated with 1/5 of 20% PEG8000/2.5M NaCl by incubating five minutes on ice. The supernatant was discarded after the solution was centrifuged at 4°C, 10 000 g for five minutes and the phage pellet was suspended in 0.5 ml TSA/BSA (chapter 11).

4.1.7 Phage quantification assay

The phage quantification assay measures a fluorescent signal from a phage solution by comparing it to a standard curve of phages at known concentrations. The method is based on two non-fluorescent probes that are used, one containing a lanthanide ion (Eu^{+}) and the other, an antenna probe. These probes have been designed to contain two different oligonucleotides that will hybridize to adjacent locations on the anti-sense strand on the chloramphenicol acetyltransferase gene in the phagemid (Figure 5). When these two components are brought into proximity they will form a fluorescent lanthanide chelate complex and the fluorescent signal will be proportional to the concentration of the phage (Lehmusvuori et al., 2012).

First, 10 µl of phage solution or the different dilutions of phages (pEB32x-scFvH) for the standard curve was mixed with 60 µl of probe mix (chapter 11). These samples were then heated to 95°C for one minute and cooled to 25°C in a PCR cycler where after, 60 µl of the samples was applied to microtiter wells (41-07TY, Kaivogen,

Finland). The plate was incubated at room temperature on a plate shaker for 15 minutes, whereafter time resolved fluorescence was measured at 340 nm using Victor 1420 Multilabel Counter (PerkinElmer, Finland). A standard curve was made from the signals received from the known phage concentrations and then the unknown phage concentration could be calculated by using it.

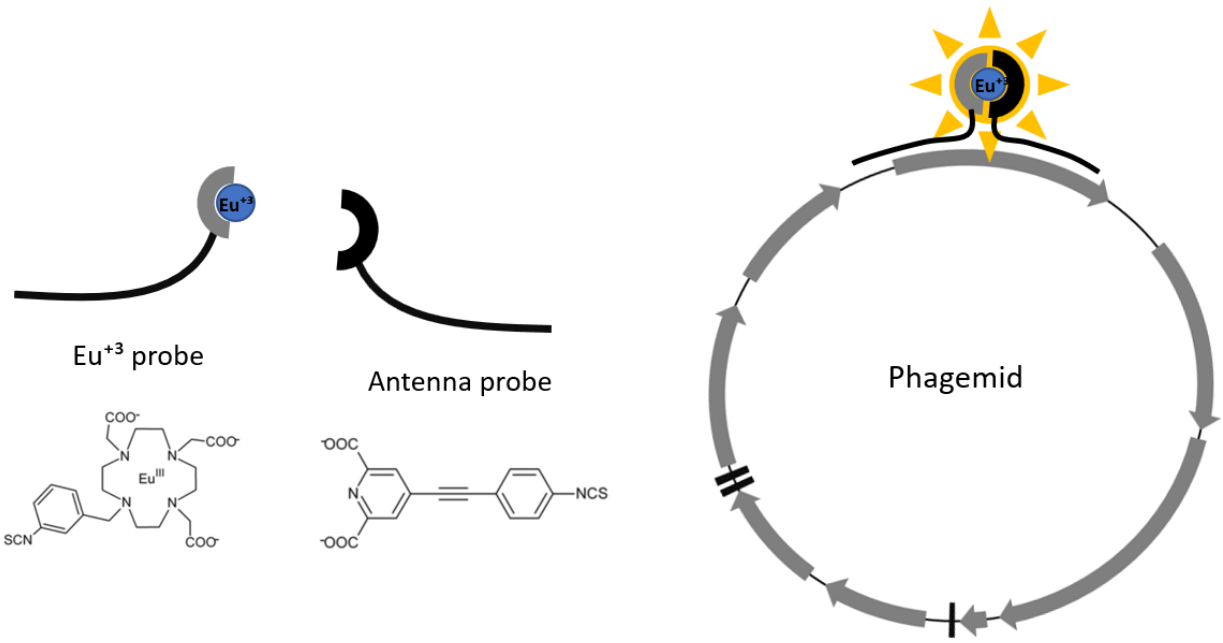

Figure 5. The principle of the phage quantification assay. The Eu^{+3} probe and the antenna probe bind to specific sites on the phagemid, which brings them close to each other enabling them to form a fluorescent lanthanide chelate complex. The strength of the fluorescent signal correlates with the amount of phagemid in the sample. The structural formulas of the Eu^{3+} chelate and the antenna ligand that are bound to the oligonucleotide probes are also demonstrated (picture modified from Lehmusvuori et al., 2012)

4.1.8 Output titrations

The measurements of the phage input and output compared to a control reaction is done to confirm that an enrichment is happening and that it is not unspecific. The enrichment of phage stocks produced after each panning round was monitored by measuring the amount of phages that were collected in each selection (the output). By knowing the output, the percentage of phages collected could be calculated by comparing to the amount of phages added into the selection (the input). In addition, a negative control reaction was done in parallel with each selection. The same amount of phages from the phage stock used for a panning round were also selected against

empty streptavidin beads that had no antigen bound to them. In the control selection the phages that bound to the beads and not the target protein were collected. The phage output is expected to increase after each selection round in contrast to the negative selection outputs. This shows that the panning rounds are not enriching phages that are unspecific binders.

The number of phages collected from each panning was calculated by phage titration. After elution a small amount of the phages from both the actual panning reaction and the control reaction were diluted in a series and these dilutions were used to infect XL1-Blue bacteria that had been grown to the logarithmic phase. The bacteria were then plated on LA-plates (0.5% glucose, 10 µg/ml tetracycline, 25 µg/ml chloramphenicol) and incubated at 37°C overnight. Chloramphenicol selection only allows the bacteria that had been infected with a phage to grow on the LA-plates. When the bacteria have been diluted enough each colony on the plates is derived from one phage-infected bacteria, so the amount of colonies on each plate was the same as the amount of phages that had been added to the bacterial culture. Concentration of the phage stock could be calculated by counting back from the phage stock dilutions.

4.1.9 Phage immunoassay

After each panning round the binding of the produced phage stock to the target of interest was determined by a phage immunoassay. For this assay a streptavidin coated microtiter plate (41-07TY, Kaivogen, Finland) was used. A Delfia Plate washer (PerkinElmer, Finland) using Kaivogen washing solution (Kaivogen, Finland) was used for the washing steps in this assay. In this assay 0.16 µg of biotinylated SSL1, 0.4 µg of biotinylated SSL5 and 0.056 µg of biotinylated SSL10 was used. The assay was performed according to the steps shown in Table 1. A Victor multilabel counter (PerkinElmer, Finland) was then used to measure the time resolved fluorescence emitted from each well. The Eu-anti-phage mAb (produced in Urpo Lamminmäki's group) that attaches to the phages that are bound to the SSL proteins is labelled with europium (Eu) (PerkinElmer, Finland). When adding the Europium intensifier solution (Kaivogen, Finland), europium will form a fluorescent chelate with the components in the enhancement solution. The fluorescent signal is directly proportional to the amount of antigen-bound phages.

Table 1 The phage immunoassay protocol used for measuring the binding of a scFv presenting phage stock to SSL proteins.

Assay step	Treatment in chronological order	Solution	Repetition and incubation
1.	Wash	Washing solution	1x
2.	Coating with biotinylated antigen	200 µl of biotinylated SSL diluted in Red Assay Buffer	30 min, RT, low shaking
3.	Wash	Washing solution	4x
4.	Phage binding	200 µl of phage diluted 1:2000 in Red Assay Buffer	1h , RT, low shaking
5.	Wash	Washing solution	4x
6.	Antibody binding	200 µl of anti-phage antibody (125 ng/ml)	1h , RT, low shaking
7.	Wash	Washing solution	4x
8.	Fluorescent chelate formation	200 µl of Europium intensifier solution	10 min, RT, low shaking

4.1.10 Plasmid construction

After the phage display was completed, the enriched genes encoding for the scFvs needed to be expressed in competent Mach1-T1 *E. coli* bacteria so that the scFvs could be tested in binding assays and later in functional assays. The gene encoding for the scFv, excluding the pIII coding gene that also was a part of the fusion protein expressed on the phage surface, was cloned into a pLK06H plasmid (Figure 6). This plasmid contains a bacterial ALP gene with a (His)6-tag at the C-terminal end.

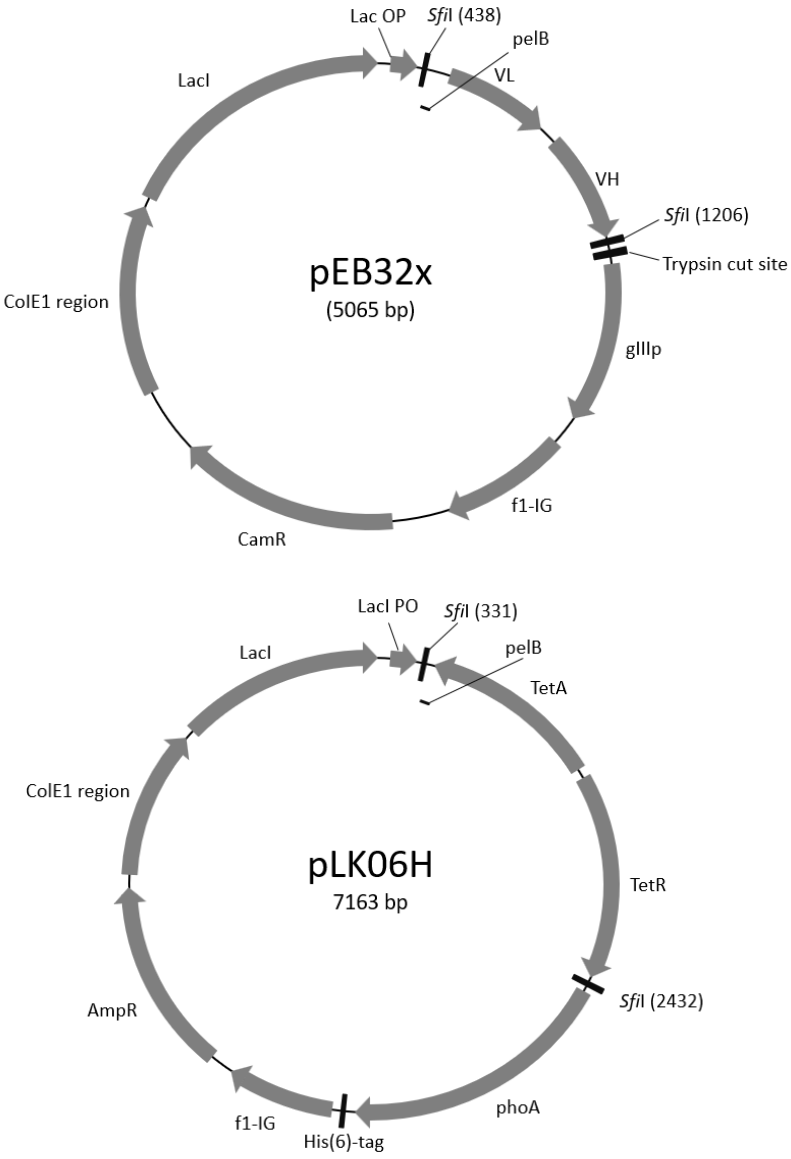

Figure 6. The pEB32x library plasmid was used in the library phages and the pLK06H was used as an expression vector in *E. coli*. The variable light (VL) and heavy (VH) chains of the scFv were cloned as a fusion protein with the phage pIII protein (gIIIp) with a trypsin cut site in between. The VL and VH are located between two *SfiI* restriction sites so that the genes can be cloned into the pLK06H plasmid with corresponding restriction sites, producing a fusion protein with alkaline phosphatases (phoA) and a His(6)-tag. Both the fusion proteins' expression is controlled by the *lac* promoter (Lac PO) that is repressed by the *lac* inhibitor (LacI). The plasmids provide the possibility for antibiotic selection with ampicillin (AmpR), tetracycline (TetR and TetA) and chloramphenicol (CamR). Marked in the map are also the M13 phage packing signal (f1-IG), the *E. coli* propagation signal (ColE1) and the periplasm signal sequence (pelB).

First a GeneJET Plasmid Miniprep Kit (Thermo Fisher, USA) was used to isolate the plasmid DNA (pEB32x, Figure 6) from the glycerol-stored cells from the last panning round. The plasmid isolate was then digested with SfiI by mixing 1 µg of plasmid with 1X Tango buffer (Thermo Fisher, USA) and 0.25 U/µl of SfiI (Thermo Fisher, USA). The digestion mixture was incubated at 50°C overnight. The vector plasmid (pLK06H, Figure 6) was digested by mixing 100 µg of the plasmid with 1X Tango buffer, 0.25 U/µl of SfiI and 0.3 U/µl of EcoO109l (Thermo Fisher, USA). This was incubated at 50°C overnight.

After the digestion, a ligation reaction was performed by mixing 20 ng of the digested vector, 42 ng of the insert, 1X T4DNA ligase buffer (Thermo Fisher, USA) and 0.25 U/µl T4 DNA-ligase (Thermo Fisher, USA). This was incubated at room temperature for one hour. The vector, that the scFv gene was inserted into, contained an ampicillin resistance gene, so that ampicillin could be used to eliminate the bacteria that were transformed with the unwanted original plasmid that only contained a chloramphenicol resistance gene.

4.1.11 Transformation

The newly constructed plasmid was transformed into a One Shot Mach1-T1 chemically competent *E. coli* cell line following the provided protocol (C8620-03, Invitrogen, USA). For each plasmid construct, 1 µl of the ligation product was added to 25 µl of the Mach1-T1 and incubated on ice for 30 minutes. Then the cells were heat shocked in a water bath at 42°C for 30 seconds. This destabilizes the bacterial membrane and makes it more permeable for DNA entry. The bacteria were placed on ice for 2 minutes, where after 250 µl of room temperature S.O.C. medium (chapter 11) was added. The bacteria were incubated at 37°C, 225 rpm for one hour and then plated on LA-plates (containing 100 µg/ml ampicillin) that were incubated at 37°C overnight.

4.1.12 ScFv-ALP protein production

The following morning, colonies were picked with tooth-picks and transferred to U-formed 96-well plates (Sarstedt, USA) with 160 µl SB medium (1% glucose, 100 µg/ml ampicillin) in each well. From each panning selection 96 individual clones, binding to either SSL1, SSL5 or SSL10, were picked. The plates were incubated at 37°C, 900 rpm, overnight.

From this overnight primary culture, secondary cultures were grown in different quantities. The primary culture was stored by freezing the bacteria at -70°C with approximately 15% glycerol. When starting a new culture from the frozen bacteria, a few microliters of bacteria were transferred to SB-media (1% glucose, 100 µg/ml ampicillin) and grown at 30°C, while shaking, overnight. Then secondary cultures could be grown by transferring a few microliters of the overnight culture to a new culture of SB-medium containing 0.05 % glucose, 100 µg/ml ampicillin. This culture was then incubated at 37°C, 900 rpm, for approximately five hours until the cultures started to become cloudy. Thereafter IPTG was added to each well in a final concentration of 0.2 mM and incubated at 26°C overnight. The temperature was kept low because it allowed for a more correct folding of the scFv protein.

4.1.13 Bacterial periplasmic extraction

To collect the scFvs that had been expressed into the bacterial periplasm, the outer cell wall needed to be lysed. This was done by adding 1/10 of 10x lysis buffer (chapter 11) and incubated at room temperature while shaking for 30 minutes. Then the cells were frozen in -70°C in order to destroy the outer membrane of the cell. For the bacteria in smaller volume in the 96-well plate the bacteria only needed to be thawed once, but for bacterial culture at a bigger volume the bacteria were frozen and thawed three times. The lysate was then collected by centrifuging the bacteria and collecting the supernatant.

4.1.14 ScFv-ALP ELISA screening assay

After the production of the scFv clones, it was possible to test their ability to bind their SSL-targets. A scFv-ALP ELISA screening assay was used to measure the binding capacity of the bacterial periplasmic lysate containing the scFv-ALPs. Alkaline phosphatase's capacity to dephosphorylate the p-nitrophenyl phosphate substrate (pNPP) was utilized (Figure 7). When pNPP is dephosphorylated, a yellow reaction product, that absorbs light at 405 nm, is formed. In the same way as in the phage immunoassay, streptavidin coated plates (41-08TY, Kaivogen, Finland) were saturated with 0.16 µg of biotinylated SSL1, 0.4 µg of biotinylated SSL5 and 0.056 µg of biotinylated SSL10 and the same washing procedures were done. The protocol shown in Table 2 was followed. Then the absorbance in the wells was measured at 405 nm with a VictorTM 1420 Multilabel Counter (PerkinElmer, Finland). The measured absorbance directly correlates with the amount of scFv-ALP fusion protein that has bound to the target on the microtiter plate.

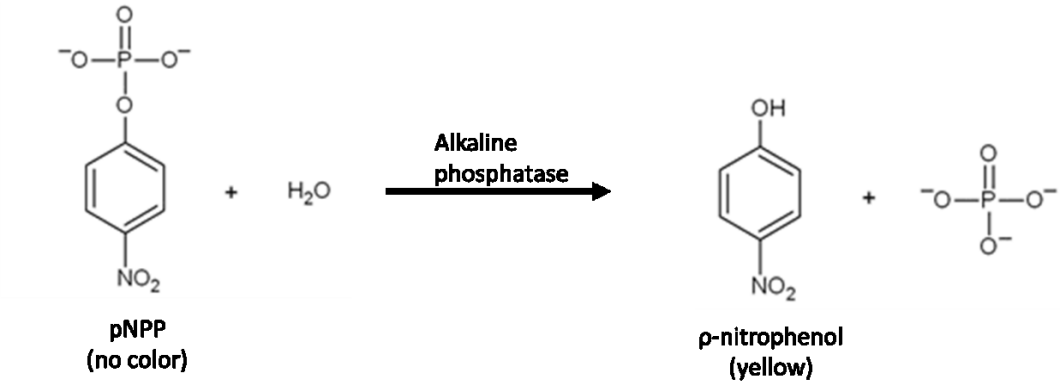

Figure 7. The chemical reaction where alkaline phosphatase dephosphorylates p-nitrophenyl phosphate.

Table 2 ScFv-ALP ELISA screening assay protocol used for measuring the binding of scFv to SSL proteins.

Assay step	Treatment in chronological order	Solution	Repetition and incubation
1.	Wash	Washing solution	1x
2.	Coating with biotiny-lated antigen	100 µl of SSL diluted in Red Assay Buffer	30 min, RT, low shaking
3.	Wash	Washing solution	4x
4.	ScFv-ALP binding	100 µl of periplasmic lysate diluted 1:5 in Red Assay Buffer	1h, RT, low shaking
5.	Wash	Washing solution	4x
6.	Dephosphorylation of pNPP	100 µl of pNPP-solution*	1h, RT, low shaking

*To make the pNPP-solution 4-pNPP-disodium salt hexahydrate (Sigma-Aldrich, USA) was diluted to 1 mg/ml in a pNPP buffer (chapter 11).

4.1.15 Sequencing

The scFvs that showed the best binding capacity were chosen to be sequenced. The Miniprep kit was used to isolate the plasmid DNA from the bacterial culture grown out of individual colonies. The DNA was sent to Macrogen Europe (the Netherlands) to be sequenced with the primer WO375 (5′-TCACACAGGAAACAGCTATGAC-3').

4.1.16 Purification of scFv-ALP proteins

After the binding of the scFv-ALP had been tested in a screening assay and the scFv genes had been sequenced, the most promising scFvs were chosen to be produced and purified so that they could later be tested in functional assays. The scFv-ALP were produced and extracted as described in the previous sections but after the extraction 8% of glycerol and 40 mM imidazol (Sigma-Aldrich, USA) was added. The scFv-ALP proteins contained a His-tag which made it possible to use HisPur™ Ni-NTA Spin Columns, 0.2 mL resin bed (Thermo Scientific No. 88224) for the purification of the protein from the bacterial periplasm lysate. The purification was done by following the producer's manual.

4.2 Functional analysis of the scFvs

4.2.1 Fluorogenic peptide MMP activity assay

With the fluorogenic peptide assay we could test whether the scFvs could interfere with the SSL1 or SSL5 proteins' ability to inhibit the MMP9 enzyme. In this assay it was possible to determine the MMP9 enzymatic activity by measuring its ability to cleave a fluorogenic peptide. This peptide contains a fluorescent 7-methoxycoumarin-4-acetyl (Mca) group and a N-3-(2,4-dinitrophenyl)-L-2,3-diamino propionyl (Dpa) group that works as an internal quencher (Neumann et al., 2004). The energy from Mca is transferred to Dpa and consequently no fluorescence is emitted. When the MMP9 cleaves this peptide the Mca group is released from the quencher and it becomes fluorescent again (Figure 8). The fluorescent signal directly correlates with the amount of active MMP9 that is present in the reaction.

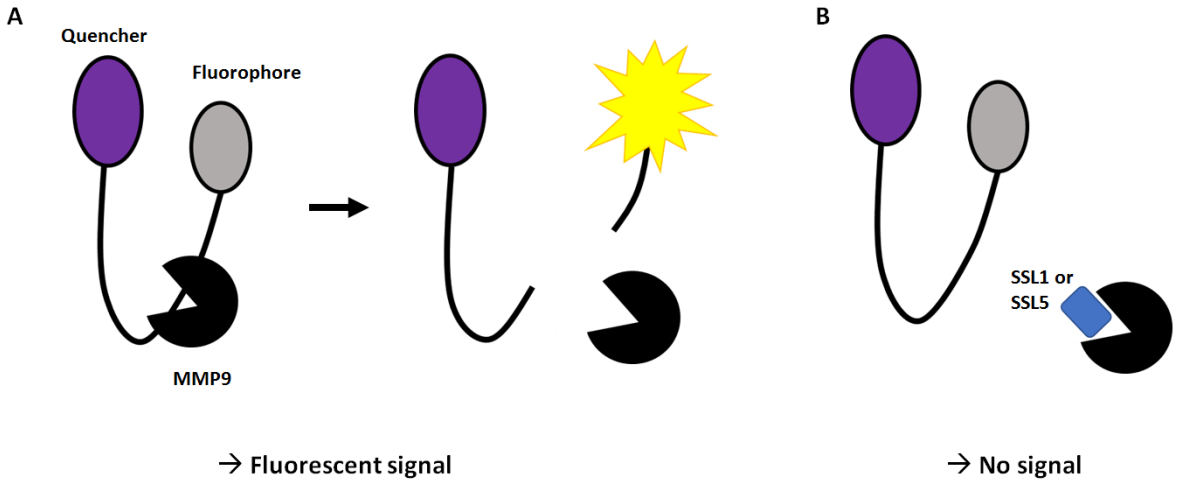

Figure 8. A fluorogenic peptide can be used to measure the enzyme activity of MMP9. The fluorogenic peptide contains a fluorophor that is connected to a quencher with a peptide link. A) When active MMP9 cleaves the peptide the fluorophor is separated from the quencher, resulting in a fluorescent signal. B) However, when SSL1 or SSL5 is added they will bind to the enzyme preventing it from cleaving the peptide and no fluorescent signal can be measured.

The first step was to activate pro-MMP9 at a concentration of 100 µg/ml by adding 10 µg/ml trypsin (Sigma-Aldrich, USA) and incubating the reaction mixture in a 37°C water bath for two hours. Thereafter the cleaving reaction was stopped by adding 100 µg/ml a1-anti-trypsin (Sigma-Aldrich, USA). SSL protein (1 µg/ml SSL1 or 0,25

µg/ml SSL5) and different concentrations of scFv were pre-incubated together for 30 minutes in a clear microtiter plate (442404, Thermo Scientific, USA) in a total volume of 25 µl. All the reactants in this assay were diluted in a fluorogenic peptide assay buffer (chapter 11). Then 25 µl of activated MMP9 was added in a final concentration of 0.25 µg/ml and incubated at room temperature for 30 minutes. Finally, 50 µl of Mca-KPLGL-Dpa-AR-NH2 Fluorogenic Peptide Substrate IX (R&D Systems, USA) was added to the reaction and immediately thereafter the fluorescence intensity was measured with a Clariostar microplate reader (BMG Labtech, Germany) at excitation and emission wavelengths of 320 and 405 nm for 40 minutes (100 cycles, 24 s cycle time). The MMP9 activity was determined by calculating the area under the signal curve that was received.

4.2.2 SDS-PAGE on MMP9-degraded collagen

To visualize the MMP9 inhibition by SSL1 and the effect of adding scFv-93, an SDS-PAGE of MMP9-degrated collagen was carried out. The enzyme was activated in the same way as in the fluorogenic peptide assay except that a trypsin inhibition cocktail (1:10 ratio) (chapter 11) was used instead of a1-anti-trypsin and the inhibition was done before using the enzyme in the assay at room temperature for 30 minutes.

In this assay SSL1 (20 µg/ml) and the scFvs were pre-incubated in a total volume of 6 µl at room temperature for 30 minutes. Then 4 µl of activated MMP9 (5 µg/ml) was added and incubated at room temperature for 10 minutes. Finally, 10 µl of collagen type I (human, Sigma-Aldrich, USA) (500 µg/ml) was added and this was incubated in a 37°C water bath overnight.

The next day 20 µl of 2X Sample buffer (with 50 mg/ml DTT) (Sigma Aldrich, USA) was added and the samples were boiled at 100°C for 3-5 min. Then 20 µl of the samples and a Precision Plus Protein Dual Color marker (Bio-Rad, USA) were loaded on a NuPAGE 4-12% Bis-Tris Gel (Invitrogen, USA) and subjected to a 180 V electrical field that separated the proteins according to their size. The gel was then stained with the Instant Blue protein stain (Expedeon, UK) and scanned with a Perfection V700 Photo Scanner (Epson, Japan).

4.2.3 PSGL-1 competitive binding assay

A PSGL-1 competitive binding assay was used to test the scFvs ability to interfere with SSL5 binding to the PSGL-1 receptor on the surface of neutrophils. The PSGL-1 receptor's natural ligand is P-selectin, but in the presence of SSL5 P-selectin binding is blocked (Figure 9). First, SSL5 (3 µg/ml) and different concentrations of scFv were pre-incubated at a total volume of 10 µl at room temperature for 15 minutes. Then 10 µl of neutrophils ($5x10^6$ cells/ml, isolated from donor blood at University Medical Center Utrecht) was added and incubated for another 15 minutes. After the addition of neutrophils the samples were incubated on ice. Then 10 µl of a P-selectin-Fc chimera (0,3 µg/ml, R&D Systems, USA) was added and the samples were further incubated for 30 minutes.

After all the treatments the cells were washed with 1 ml RPMI 1640 buffer with HEPES and human serum albumin (Gibco, USA). Then, in order to visualize the amount of P-selectin-Fc that had bound to the PSGL-1 receptor, 50 µl of Fc binding Donkey-anti-human-IgG(H+L)-APC (Jackson Immuno Research, UK), diluted to a 1:400 ratio, was added and incubated for 30 minutes. Again, the cells were washed with 1 ml buffer after which the fluorescent signal from the antibody was measured with a FACSVerse flow cytometer (BD Bioscience, USA). The neutrophils were selected by gating and the results were analyzed using the FlowJo software (FlowJo, USA).

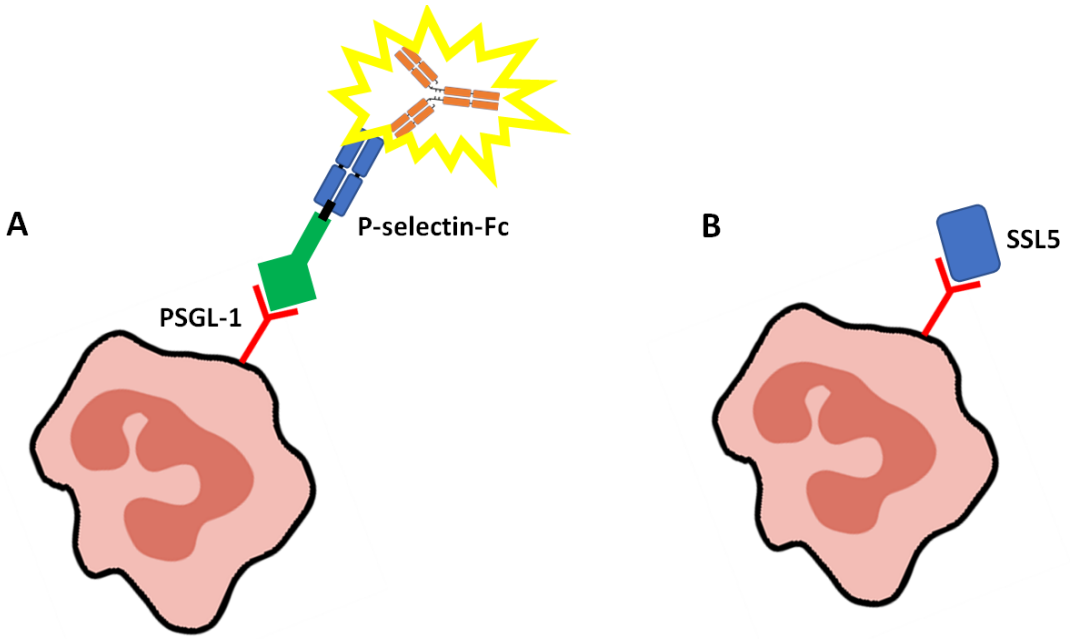

Figure 9. The competitive binding of P-selectin and SSL5 to PSGL-1 was measured with a fluorescent antibody. Neutrophils naturally express PSGL-1 on their surface. A) A P-selectin-Fc chimera protein binds to PSGL-1 and an Fc-binding fluorescent antibody makes it possible to detect the amount of neutrophils with P-selectin bound to them. B) When SSL5 is present it will bind to PSGL-1, consequently inhibiting P-selectin-Fc and the fluorescent antibody from binding. The lack of fluorescent signal indicates that P-selectin has not bound to PSGL-1 on the neutrophil.

4.2.4 DNA gel retardation assay

In order to test if the scFvs could intervene with SSL10 binding to DNA, a DNA retardation assay was performed. In this analysis we could determine whether SSL10 is bound to DNA by examining the size differences on a DNA retardation gel. In this assay a cruciform type of DNA was used because it runs at a suitable height on the gel. The cruciform DNA is made up out of four different oligonucleotides that are partially complementary with each other and will form a cruciform structure when they are annealed (Figure 10). This was done by heating up a 1:1:1:1 molarity mixture of the oligonucleotides to 70° C and then slowly cooling it down to 25°C, over a two-hour period.

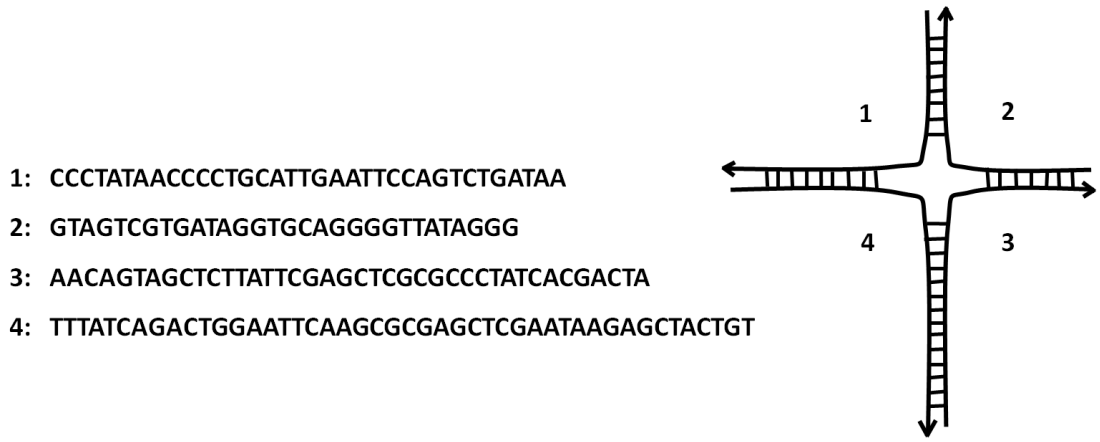

Figure 10. Four different oligonucleotide strands that are partially complementary, enabling them to assemble into a cruciform structure. The four oligonucleotides (shown on the left) will anneal together as demonstrated on the right. However, in solution the molecule is most likely tetrahedral instead of being on one plane as it is represented here (modified from Bianchi, 1988).

Next, 1 µl of SSL10 (100 µg/ml) and 7 µl of of scFv (72 µg/ml) were pre-incubated at room temperature for 15 minutes. Then 1 µl of the cruciform DNA was added and this was further incubated for 10 minutes. Finally, 1 µl of Novex Hi-Density TBE Sample Buffer (Invitrogen, USA) was added. The samples were then applied to a 6% DNA Retardation Gel (Invitrogen, USA) with a 100-bp ladder and run at 100 V in 0.5x TBE buffer (Invitrogen, USA). The gels were then stained in 0.5x TBE buffer containing 1:10000 SYBR Safe (Thermo Fisher, USA) for 15 minutes and scanned with a Gel Doc XR+ scanner (Bio-Rad, USA).

5 Results

5.1 ScFv-displaying phages were enriched against all three SSL targets

ScFv antibodies were selected against SSL1, SSL5 and SSL10 with phage display from synthetic antibody libraries scFv M and P (Huovinen et.al., 2013). In order to monitor the phage display process and predict that the enrichment process was proceeding as planned, output titrations and phage immunoassays were performed for each panning round. After each panning round the phage stock produced, should contain a larger fraction of phages binding to the specific target compared to the previous phage stock. In contrast, the background should stay low and decrease after each panning round. The phage immunoassay was carried out to test if the produced phage stocks were showing binding to their specific target.

In all three selection experiments (against SSL1, SSL5 and SSL10) the output (Figure 11) and the enrichment factor (Table 3) increased after each panning round. In the first round of selections a larger number of phages 5 x 10^{12} cfu was used. The number of phages should be around a hundred times greater than the actual library size, so that in theory all the different phage clones are entered into the selection. The outputs from the first selection round showed that less than 0,001% of the phages were recovered (Figure 11). This was to be expected because only a fraction of the phages in a library will bind to a specific target. When specific phages were enriched the output/input ratio increased and percentage of background decreased (Table 3). Enrichment was also seen as increase in signal in immunoassay measuring phage binding to the target antigen. After three rounds of panning, specific binding to all three targets were observed in the phage immunoassays and the phage stocks gave rise to a higher signal after each panning round (Figure 12).

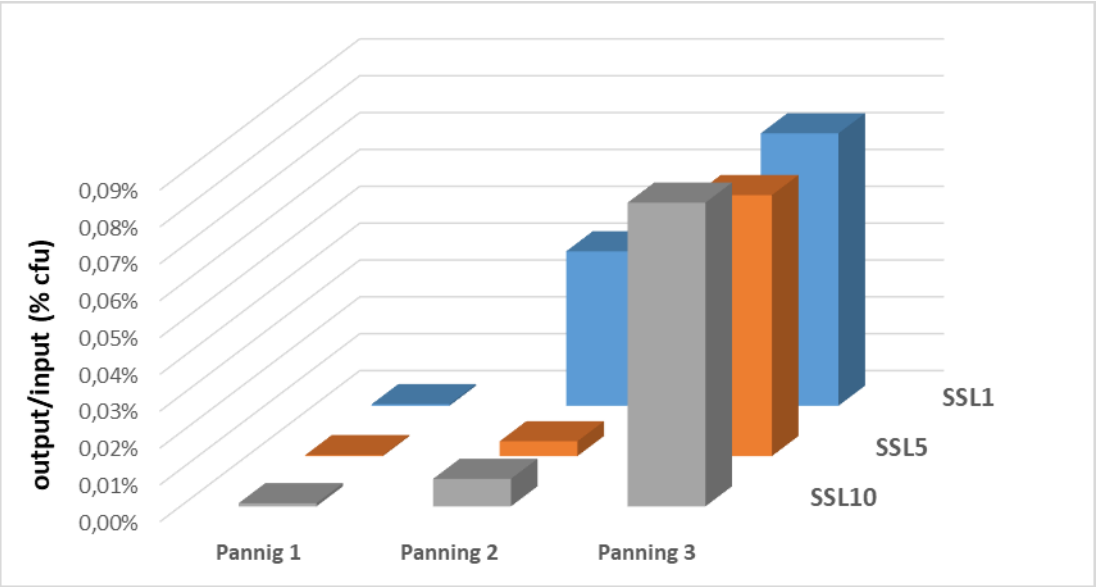

Figure 11. The percentages of phages collected after three rounds of panning against SSL1, SSL5 or SSL10 with scFvM2 and scFvP library. The percentage is calculated by dividing the amount of phages eluted after a selection with the amount of phages added to the selection. The number of phages in the elution was measured by making dilution gradients of the phage-infected cell cultures.

Table 3. Selective enrichment of phages by panning against proteins SSL1, SSL5 and SSL10.

	SSL1			SSL5			SSL10		
Panning round	**Output/ input[A]**	**Enrich-ment[B]**	**Back-ground[C]**	**Input/ output**	**Enrich-ment**	**Back-ground**	**Input/ output**	**Enrich-ment**	**Back-ground**
1	0.00044%	1	-	0.00025%	1	-	0.00086%	1	-
2	0.042%	96	1.0%	0.0040%	16	16.2%	0.0075%	9	5.5%
3	0.074%	170	0.0020%	0.071%	283	0.0081%	0.082%	96	0.015%

[A]The percentage of phages that were collected (output) from the total amount of phages that were used in the selection reaction (input). [B]The enrichment factor was calculated by comparing the second and the third round to the first round. [C]The percentage of the collected phages that presented unspecific binding (background).

5.2 The phage display targeting SSL1 produced a strong and specific enrichment

After each panning the output is expected to at least increase by 10-fold in relation to the previous round, although it might not always be achievable in the earliest rounds (Frei et al., 2016). The total enrichment from the first to the last panning round resulted in a 170-fold enrichment against the SSL1 target (Table 3). Already between the first and the second selections a very high enrichment ratio of 96 was generated. The control output indicated that the phage stocks produced to bind SSL1 were very specific as the background accounted for only 1.0% of the phages in the second selection and decreased further in the following round. The SSL1 selection also performed well in the phage immunoassays. After the first panning round the phage stock produced against SSL1 already gave a high signal towards SSL1with a low background signal and no affinity for the two other SSLs (Figure 12). In the two following panning rounds the results of high specificity and low background continued.

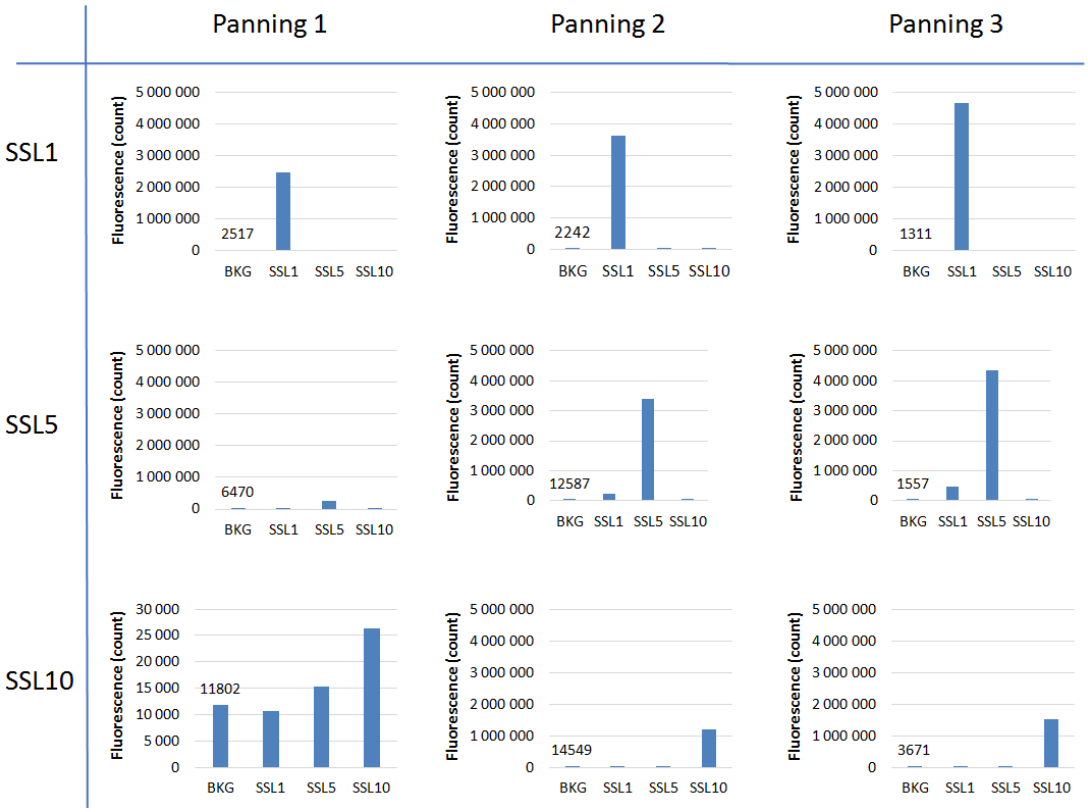

Figure 12. Binding profiles of the phage stocks that were produced in three panning rounds against SSL1, SSL5 or SSL10. A phage immunoassay was used to determine the binding of the panned phage stocks to all three SSL proteins (SSL1, SSL5, SSL10) and the background (BKG) being wells without any antigen. The results for the selections done against each antigen is shown horizontally and the panning round is shown vertically. All the graphs are in the same scale except for panning 1 of SSL10, in order to better visualize the background.

5.3 The phage display targeting SSL5 generated a strong enrichment that was less specific

The SSL5 selection produced the highest and the steadiest enrichment as the enrichment was 16-fold between the first and second panning round and 18-fold between the second and the third (Table 3). Ultimately leading to a total enrichment ratio of 283. However, in the second panning round the background was as high as 16.2%, but it decreased drastically down to 0.0081% in the third round. In the binding assays the phages produced against SSL5 showed strong binding for SSL5, but also showed some binding to SSL1 (Figure 12). After the final panning round the affinity for SSL1 was 10.9% of the signal measured for SSL5.

5.4 The phage display targeting SSL10 produced a weaker enrichment with a higher background binding

The selection targeting SSL10 did come close to achieving a total of 100-fold enrichment between the first and the third panning round. It only produced a 9-fold enrichment between the first and the second round, but the following round the 10-fold enrichment was achieved. This resulted in a total enrichment ratio of 96 between the first and the third selection. The control output titrations indicated a moderately high background binding in the second panning round with 5.5%, but it reduced to 0.015% in the third round. After the first selection round the phage stock produced to bind to SSL10, showed little specificity to SSL10 as the background accounted for almost half of the signal (Figure 12). The signal only reached to about 25 000 counts, compared to the signal of the other two SSL binding phage stocks being 10x (SSL5) and 100x (SSL1) higher. After the two following panning rounds the background

signal decreased and the binding to SSL10 increased to over 1 x 10^6 cps, but never reached as high as the signals for the two other selections.

5.5 Several target-binding scFvs were produced for all three phage display targets

For each of the three selections, 96 scFv-ALP clones were produced by expression into *E. coli* periplasm. The binding of the scFv-clones against all of the three SSL-targets were tested in a scFv-ALP ELISA screening assay (Supplemental figure 1). Based on these results between ten and twenty of the scFvs were chosen for production in a larger scale.

When a larger amount of the chosen scFvs had been produced, a new scFv-ALP ELISA screening assay was performed on this lysate to confirm the binding activity and specificity. All of the SSL1 binders showed a high signal, whereas the signal for the SSL5 and SSL10 binders was lower in comparison (Figure 13). The scFvs that gave a signal lower than 0.4 were discarded. Interestingly, two of the scFv-fragments that had been produced by panning against SSL5 showed binding affinity against SSL1, but as they showed such a low binding signal they were also discarded.

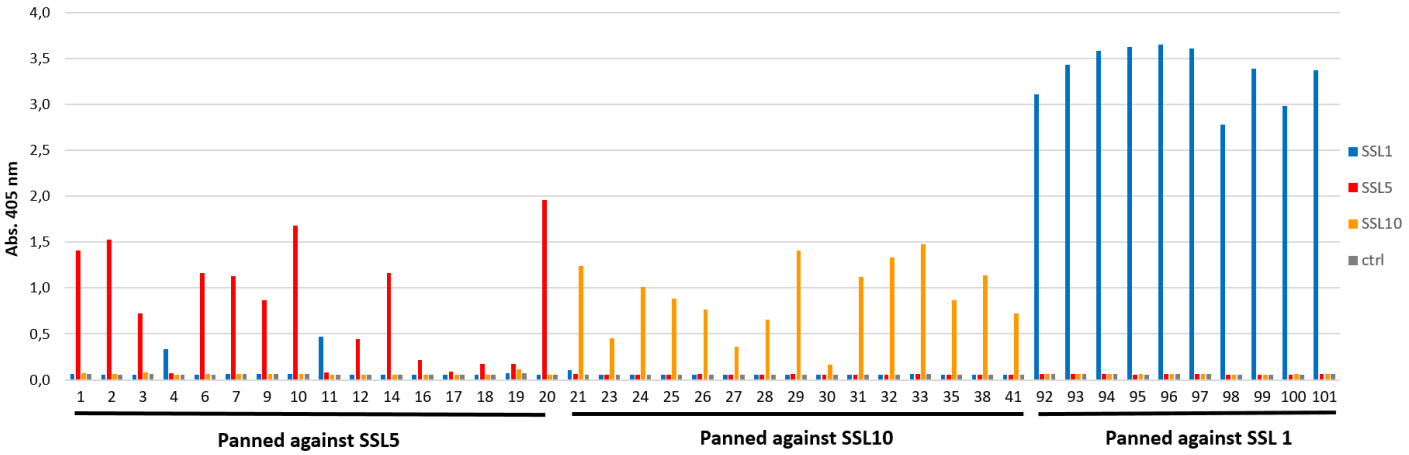

Figure 13. ScFv-ALP protein binding to SSL1, SSL5 and SSL10. The binding of the scFvs was measured against SSL1 (blue), SSL5 (red), SSL10 (yellow) and a control sample (ctrl) without any antigen (grey).

5.6 The SSL1-targeted binders had the most homogenous CDR-H3 loops

Based on the scFv-ALP ELISA screening assay results a number of scFv-fragments were chosen for further testing in functional assays. The genes corresponding to these scFv-fragments were sequenced and then translated into the amino acid sequences and aligned, so that they could be compared. Out of all the sequences there were two pairs of scFvs that had identical CDR-H3 loop in the SSL1 and one pair in the SSL5-binding clones. However, the two pairs of SSL1-binding scFvs had quite a few differences in amino acids in the CDR-H1 and H2 regions, whereas the SSL5-binding scFvs had identical sequences in the two regions.

Comparing the sequences within all the three selections, the selection against SSL1 clearly resulted in a more cohesive array of CDR-H3 sequences, with all of them being seven amino acids in length and only two or three amino acids varying between them. In contrast the outcome of selections against SSL5 and SSL10 were an assortment of sequences varying between 6-18 amino acids in length (Table 4).

Table 4 Information about the CDR-H3 amino acid (aa) sequences of the scFvs-fragments produced against SSL1, SSL5 and SSL10.

	SSL1	**SSL5**	**SSL10**
ScFvs sequenced	9	18	19
Unique CDR-H3 sequences	7	17	19
CDR-H3 lengths	7 aa	6-18 aa	8-17 aa

5.7 SSL5-binding scFvs hardly inhibit the SSL5 activity on MMP9

Since both SSL1 and SSL5 have been shown to inhibit MMP9 enzymatic activity (Koymans et al., 2016), both the SSL1 and SSL5 binding scFvs were tested in a fluorogenic peptide assay to see if they had a recovering effect on the MMP9 activity by binding to the SSL proteins. In total, nine SSL1 and ten SSL5 binding scFvs were tested. Initially, a higher concentration of scFv was used to determine if any recovering effect on MMP9 could be observed (Supplemental figure 2 and 3). The scFvs that showed enough effect were then titrated in the fluorogenic peptide assay to determine the concentration that still could recover the MMP9 enzymatic effect.

Seven of the SSL5-binding scFvs that had shown the largest recovering effect on MMP9 in the previous MMP9 assays were titrated (50 µg/ml to 0.63 µg/ml). At the highest concentration of 50 µg/ml (670 nM) the addition of scFvs showed 20-40% recoveries of the MMP9 activity, with scFv-14 and 20 showing the highest recovery (Figure 14). However, by lowering the concentration to 10 µg/ml (130 nM) already dropped the effect to a non-significant level.

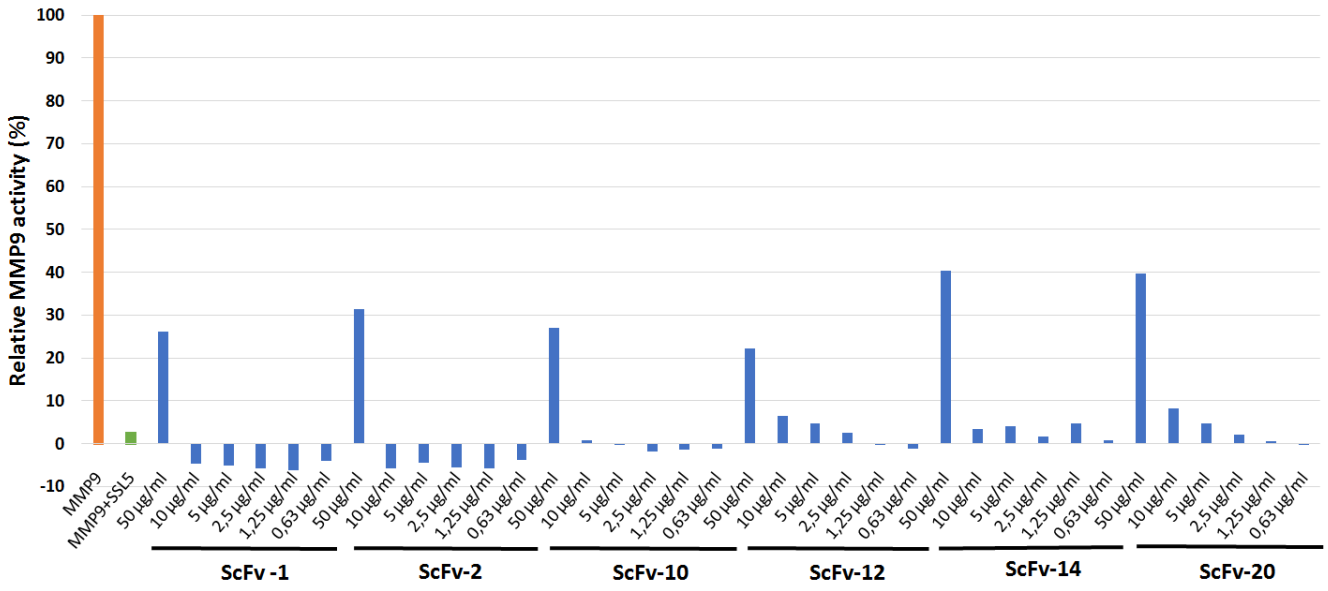

Figure 14. The recovery of SSL5-inhibited MMP9 activity by SSL5-binding scFvs. The uninhibited MMP9 activity (orange) was set to 100% and the activity adjusted in relation to this. The 0.125 µg/ml (4.5 nM) SSL5 inhibited MMP9 is shown in green and the addition of a concentration range (50 µg/ml-0.63 µg/ml) of scFv is shown in blue.

5.8 ScFv-93 potently counteracts the SSL1-mediated inhibition of MMP9

When testing the SSL1-binding scFvs, four scFvs were of interest, based on the previous results. The MMP9 activity was inhibited with 0.5 µg/ml (19 nM) SSL1 and the scFvs were titrated from (50 µg/ml to 0.63 µg/ml). Four scFvs showed a high recovery between 75 to 90% at the highest concentration (670 nM) (Figure 15). Lowering the concentration to 10 µg/ml (130 nM) dropped the effect of scFv-98 to 30% whereas for scFv-95 and 96 still stayed around 75%, however further dilution also dropped the effect under 35%. ScFv-93 showed the highest effect of them all. Dilution down to a concentration of 2.5 µg/ml (33 nM) kept the effect at over 90% and a concentration of 1.25 µg/ml (16 nM) still showed a 50% recovery of the MMP9 activity.

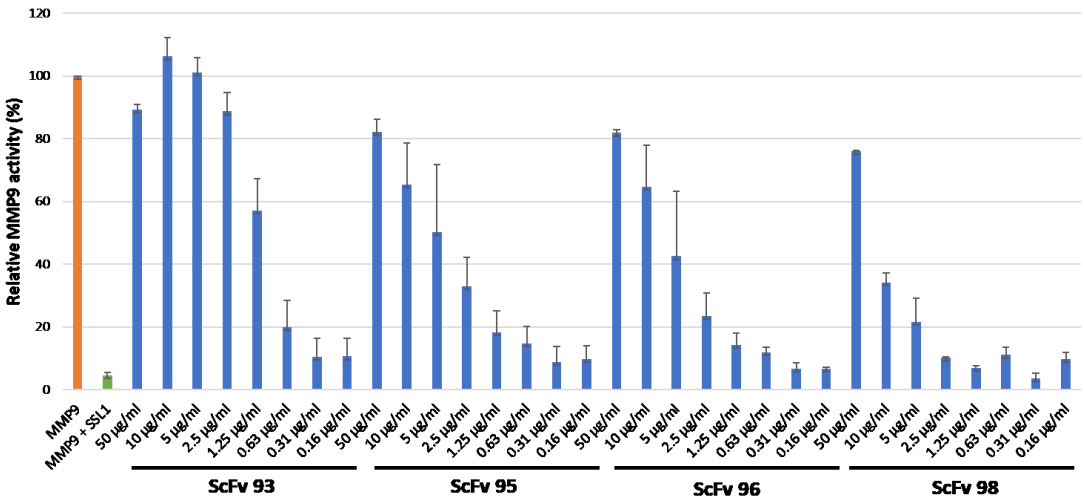

Figure 15. The recovery of SSL1-inhibited MMP9 activity by different concentrations of SSL1-binding scFvs. The uninhibited MMP9 activity (orange) was set to 100% and the activity adjusted in relation to this. MMP9 activity when inhibited by (0.5 µg/ml) SSL1 is shown in green and the addition of scFv at different concentrations is shown in blue. The first four scFv-fragments in the figure were titrated between 50-0.16 µg/ml. The results represent a mean of n=2 ± standard deviation.

5.9 ScFv-93 also seems to restore some of the MMP9 enzymes ability to degrade collagen I

In order to test whether scFv-93 would have an effect on SSL1-inhibited MMP9 activity in a more natural system compared to the fluorogenic peptide assay, the MMP9 activity was also measured by assessing how well it breaks down collagen I. In this experiment, collagen I was incubated overnight with activated MMP9 in the absence or presence of SSL1 and scFv-93. The collagen degradation was then visualized on SDS-PAGE.

The lane containing only collagen has two visible lines at a little under 150 kDa and a line a little above 250 kDa and some that are even bigger (Figure 16). When adding MMP9 these lines disappear completely, but the addition of 3.0 µg/ml (120 nM) SSL1 inhibits the collagen degradation revealing the same lines as in the nondegraded collagen. In the following four lanes scFv-93 was titrated from 30 µg/ml to 3.75 µg/ml. The scFv-93 does not completely restore the cleaving capacity of inhibited MMP9, because some faint lines can still be identified, but at the concentration of 30 µg/ml (400 nM) and 15 µg/ml (200 nM) the two lines around the size of 130 kDa are evidently not as strong as in the lane with MMP9 and SSL1. The collagen lines get stronger as the scFv-93 is reduced and at the concentration of 3.75 µg/ml (50 nM) no effect can be detected. The bands in the last lane also indicate that scFv-93 has no effect on the SSL5 proteins ability to inhibit MMP9.

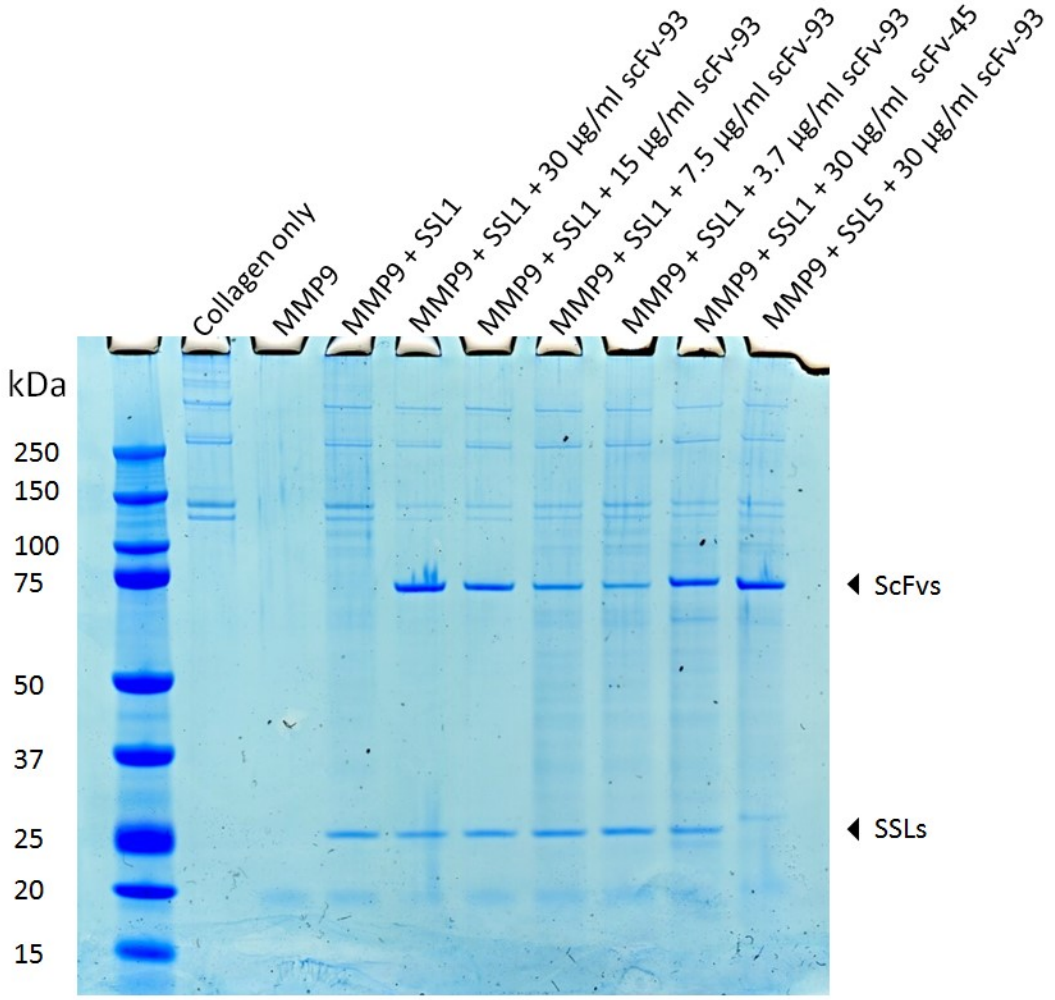

Figure 16. The effect of scFv-93 on SSL1-inhibited MMP9-mediated collagen degradation, visualized on an SDS-PAGE-gel. The first lane contains a protein size marker, the second lane only contains collagen and the third lane shows how MMP9 digests the collagen. In the fourth lane the MMP9 inhibition by SSL1 (3.0 µg/ml) is shown and then the effect of adding different concentrations (3.7-30 µg/ml) of scFv displayed in the following lanes. In the last two lanes the non-binding scFv-45 and SSL5 instead of SSL1 were used as controls.

5.10 The scFvs had little effect on SSL5-mediated PSGL-1 inhibition

We decided to test if the SSL5-binding scFvs were able to inhibit another of the SSL5 proteins functions. SSL5 inhibits P-selectin from binding to its principal ligand PSGL-1, by binding to the ligand. This interaction was investigated with a PSGL-1 competitive binding assay. A 1.0 µg/ml (36 nM) concentration of SSL5 inhibited 72.7% of the P-selectin binding to PSGL-1 on the surface of neutrophils (Figure 17). The addition of scFv-proteins exhibited little to no recovery of P-selectin binding. ScFv-12 showed the strongest effect with 59.5% P-selecting binding, which means that 44.2% of the SSL5 effect was inhibited. This was however at a very high concentration of scFv (50 µg/ml which is 670 nM).

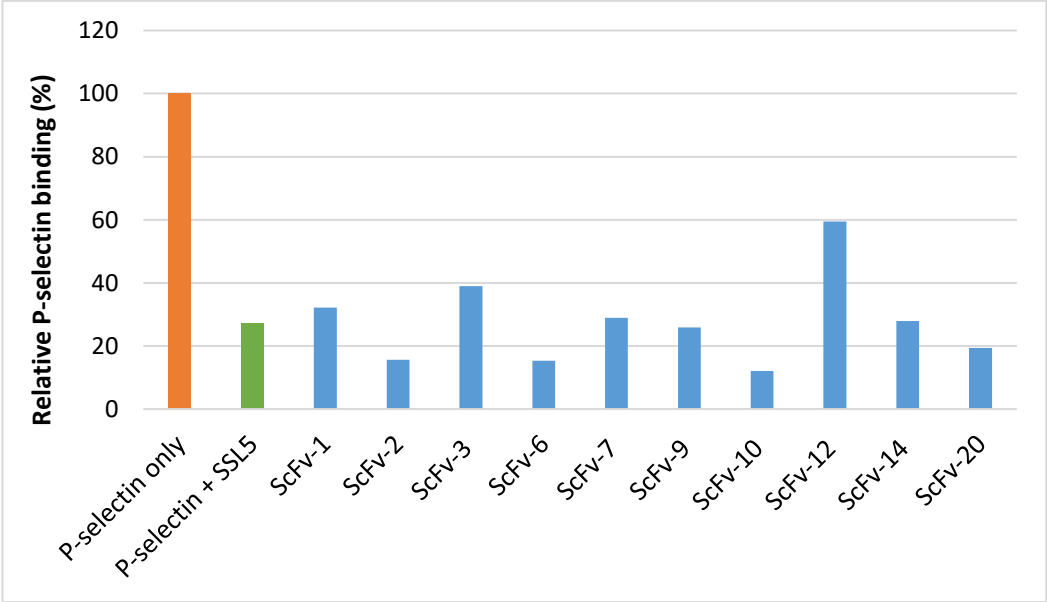

Figure 17. ScFvs effect on P-selectin binding to PSGL-1 in the presence of SSL5. P-selectin binding to PSGL-1 was measured after the addition of SSL5 (1.0 µg/ml) (green) and scFvs (50 µg/ml) (blue). A control of only P-selectin was also measured (orange) and the rest of the signals were adjusted in relation to this.

5.11 ScFvs had no effect on SSL10 binding to DNA

A DNA retardation assay was used to determine whether the SSL10-binding scFvs would have any effect on SSL10 binding to DNA. In this assay the binding of SSL10 to cruciform DNA was visualized simply by separating the DNA by size on a DNA retardation gel. Cruciform DNA alone migrated the furthest on the gel and the addition of SSL10 resulted in the DNA shifting higher up (Figure 18). Almost all the lanes

containing 10 µg/ml (430 nM) SSL10 and cruciform DNA have one band that is higher than the band produced by only DNA. In addition, some of the lanes have a second faint band slightly higher up. A HU-protein that binds double stranded DNA was used as a positive control for DNA binding. The cruciform DNA together with the HU protein generated two clear bands of DNA that were around the same height as the bands in the lanes containing SSL10.

None of the scFvs, at a 50 µg/ml (670 nM) concentration, were completely able to shift down the DNA bands to the height where only cruciform DNA was used (Figure 18). However, the addition of some of the scFvs (21, 33, 23, 28, 80, 82, 35) resulted in a faint band at the same height as cruciform DNA only, in addition to a band at the height of cruciform DNA together with SSL10. ScFv-32 gave rise to supershifts of the DNA, where two bands of DNA were detected much higher up on the gel.

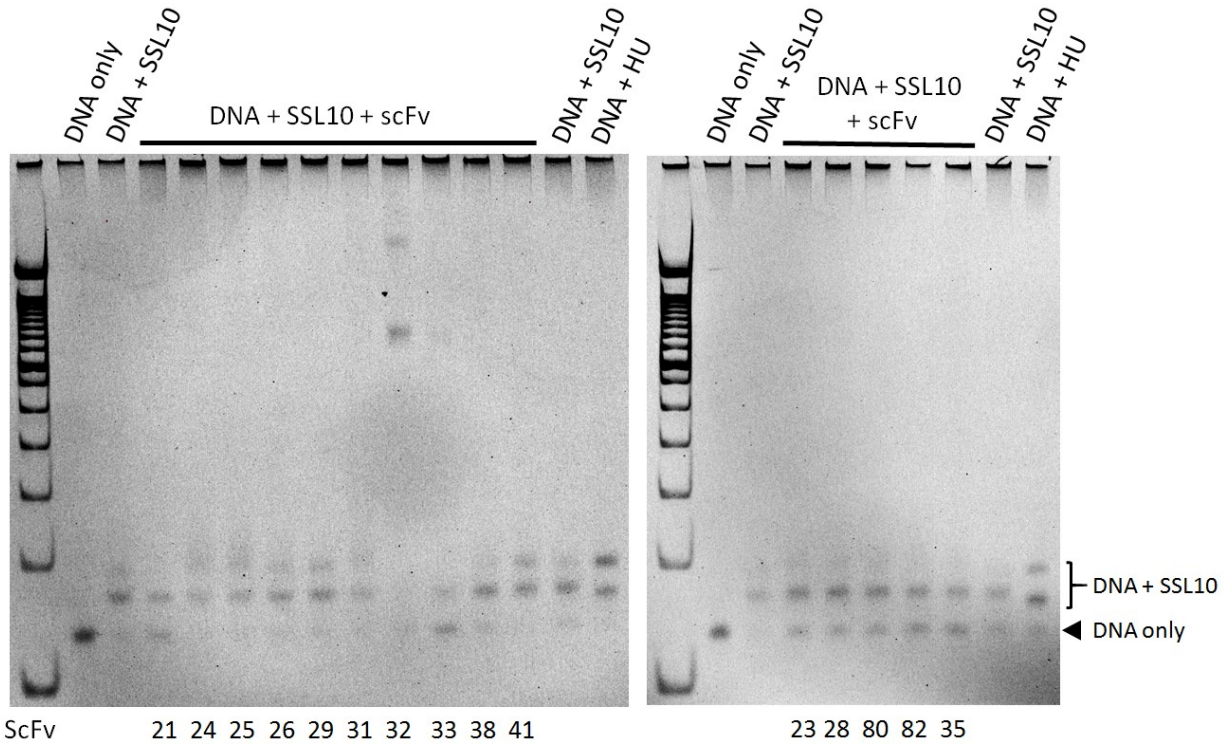

Figure 18. SSL10 binding scFvs were unable to prevent SSL10 from binding to cruciform DNA. A DNA retardation assay was used to visualize the SSL10 binding to cruciform DNA. The size difference in lanes containing cruciform DNA only and the addition of (10 µg/ml) SSL10 was compared in the second and third lanes of the gels. In the last two lanes cruciform DNA with SSL10 or HU-protein were compared. In the lanes between, the effect of the different scFvs (50 µg/ml) on SSL10 and DNA binding was measured. A 100-bp ladder was used as a marker in the first lane of the gels.

6 Discussion

Antibiotic resistant bacteria are an ever increasing and major problem in the world today. The methicillin-resistant strains of *S. aureus* are currently among the leading causes of antibiotic resistance (Lakhundi et al., 2018). The bacteria's ability to become resistant to different types of antibiotics encourages research into alternative strategies to treat infections. The bacteria have also developed different strategies to evade the host's immune system in order to promote survival and successful transmission to the next host. Research into these mechanisms is important in order to develop strategies to restore the capacity of the immune system and thereby allowing more efficient clearance of the bacteria and preventing further infections. We decided to explore the possibility to produce scFv-fragments targeting three of *S. aureus* immune evasion proteins: SSL1, SSL5 and SSL10. We then proceeded to test if the antibody fragments with observed binding affinity towards these SSL proteins could be used to prevent some of their immune modulating actions.

6.1 The phage display successfully enriched scFv-binders against SSL1, SSL5 and SSL10

Our first goal in this project was to produce scFv-fragments that bind to SSL1, SSL5 or SSL10 using the phage display technique. The output titrations and immunoassays indicated that three rounds of panning were enough to enrich scFv-presenting phages towards all three targets. Both the output titrations and the phage immunoassays showed that the enrichment of binders became stronger for each round of panning and that the unspecific binding decreased at the same time (Table 3 and Figure 12). Even if the amount of background binding varied between the three selections at different points of the phage display, by the time the third panning round had been completed the background had dropped to a minimal level. The cloning and expression of the scFvs as fusion proteins with ALP was successful as majority of the clones showed binding to their target (Supplemental figure 1).

6.2 The selection with SSL1 as a target most produced the best binders

At every step of the way the SSL1-binding scFvs seemed to perform the best in the different experiments. In the output measurements the enrichment was high at the

same time as the background binding was very low, and in the phage immunoassay the binding to SSL1 was clear and specific from the first panning round, again the background being very low (Table 3 and Figure 12). When comparing to the selections made against SSL5 and SSL10, they had higher background binding during the first two panning rounds. However, it decreased to a similar level as the SSL1-selection after the third and final panning round. Also, the screening results showed that a larger proportion of the SSL1 binding clones gave a higher signal, compared to the SSL5 and SSL10 binding scFvs (Supplemental figure 1). These binding results do not necessarily imply that the scFvs bound their target with low affinity, because the concentration of scFv can differ between the clones and some can have an advantage if they are more sufficiently expressed. However, the screening assay can still give some indication of this.

There can be many reasons to why the results of a phage display can vary. In a phage library only a small proportion of phage clones will be able to bind a specific target and only some of them will be high-affinity binders. The numbers will fluctuate depending on how well the library suits the target. There will always be a factor of coincidence involved, as a certain number of clones are picked at random to be further investigated. However, one possibility is that there might have been some differences in how optimal the biotinylation of the SSL proteins was or in how much of the protein was used for the selection. If the proteins are too heavily biotinylated it might block scFv binding sites on the proteins surface and might lead to inactivation of the protein, particularly if any basic amino acids are present at the active site (Chambers et al. 2010). It is important to find a balance in the density of antigen on the immobilized target. A higher density decreases the amount of background binders, but it reduces the selection stringency at the same time (Vodnik et al., 2011).

6.3 One scFv strongly bound to and was able to inhibit SSL1 from interfering with MMP9 activity

ScFv-93 was at a low concentration able to prohibit SSL1 from inhibiting MMP9 when tested in a fluorogenic peptide assay. A concentration as low as 0.033 μM of scFv-93 showed an over 90% recovering effect on the MMP9 enzyme when it was inhibited by 0.019 μM SSL1 (Figure 15). This means that a molar ratio of 1.7:1 of the scFv in

relation to SSL1, was still sufficient enough to show an effect. One scFv only has one antigen binding site, which means that one scFv-93 should be able to bind to and inhibit one SSL1 protein. This entails that the ideal molar ratio for inhibiting SSL1 would be 1:1. When the concentration of scFv-93 was lowered to a molar ratio that was below 1:1, its recovering effect decreased, as would be expected. These results indicate that scFv-93 has a strong affinity to SSL1 and is quite efficiently able to inhibit SSL1 from interfering with MMP9.

The fluorogenic peptide assay is an efficient way to measure MMP enzymatic activity, but we wanted to examine if the scFv-93 is also able to inhibit SSL1 from hindering MMP9 activity in a more natural setting. Thus, scFv-93s capacity to inhibit SSL1 was further examined by visualizing MMP9 mediated collagen I degradation in an SDS-PAGE. The SDS-PAGE revealed that scFv-93 had some recovering effect on the MMP9 collagen degradation (Figure 16). A 200 nM concentration was able to inhibit 120 nM SSL1 enough for there to be an observable difference in collagen degradation. Which suggests that scFv-93 also might be able to have a recovering effect on collagen degradation by SSL1-inhibited MMP9. However, the MMP9 activity could not be recovered completely as there were still some faint bands left in these lanes, compared to the lane with MMP9 only where collagen was completely degraded. In this assay, 400 nM was the highest concentration of scFv-93 that was tested which is a 3.3-fold molar excess of scFv. It would be interesting to test an even higher concentration of scFv-93 to examine if a complete collagen degradation could have been achieved.

In addition to these results, Assistant Professor Dr. Carla J.C. de Haas used gel filtration chromatography to study the binding of scFv-93 to SSL1. The results confirmed that the proteins formed a complex (Supplemental figure 5). This further validates that scFv-93 binding to SSL1 accounts for the results we acquired. However, further studies and repetitions still need to be performed in order to determine how well scFv-93 can restore the MMP9 activity in the presence of SSL1.

6.4 The SSL5 and SSL10 binding scFvs were unable to inhibit the examined SSL functions

We were not able to show a meaningful inhibition of the SSL5 or SSL10 protein with the scFvs that were produced to bind them. The ability of scFvs to interfere with the SSL5 protein's interaction with either PSGL-1 or MMP9 was tested as well as the ability to prevent SSL10 from binding DNA. Additionally, an assay where the SSL10 protein's inhibitory action on *S. aureus* phagocytosis by neutrophils was used to examine the scFvs. This experiment was performed by MSc Ida Alanko (Supplemental figure 4). None of these experiments were able to demonstrate a strong enough effect of the scFvs that were produced.

However, it is important to note that these experiments tested very specific functions and do not exclude the possibility of the scFvs from having an impact on other functions that the SSL proteins might have. Likewise, the results do not indicate that the scFvs are not able to bind to their target. The scFvs may still have a strong affinity for epitopes that are not involved in the SSLs functions. In the DNA retardation assay scFv-32 generated a supershift of the DNA. This is likely due to the scFv being bound to SSL10 in addition to the DNA binding. This illustrates that the scFv is binding to its target without intervening with its function. Also, because of the lack of time we were not able to repeat the experiments as many times as we would have preferred. We only had the chance to perform a few experiments and had to abandon any unpromising leads to pursue the more promising alternatives.

6.5 Could scFv-93 prevent SSL1 from inhibiting other MMPs?

MMPs seem to have an important role in fine-tuning the immune response against a pathogenic infection, especially in initiating the response and in tissue remodeling (Elkington et al., 2005). To weaken the MMPs' immune defense modulating effect, *S. aureus* has evolved into producing SSL1 and SSL5 that are able to inhibit at least eight different MMPs (MMP1, 2, 7-9, 12-14) (Koymans et al., 2016). The scFv-93 that we produced was to some extent able to prevent SSL1 from inhibiting MMP9.

This sparks the question whether the scFv further could interfere with the interaction between SSL1 and other MMPs. The MMPs exhibit a high sequence similarity of their

catalytic domain, ranging between 56-64% (Lukacova et al., 2003). Because of this sequence similarity it might be possible that SSL1 uses a conserved system to inhibit some of the other MMPs, suggesting that scFv-93 could possibly also suppress this interaction. Lukacova et al. (2003) investigated the interaction energies between all the MMPs and five different chemical probes, and then compared the properties of the MMP binding sites as a pairwise correlation with each other. The results suggested that the MMP9 binding site was the most similar with MMP2, 3, 7, 8, 12 and 14. Out of these, all but MMP3 were shown to be inhibited by SSL1 (Koymans et al., 2016). Therefore, it would be interesting to start by testing scFv-93s ability to prevent SSL1 from inhibiting MMP2, 7, 8, 12 or 14. This hypothesis could be explored by using the same fluorogenic peptide MMP activity assay that was used in this project.

More knowledge about how SSL1, as well as SSL5, interact with MMPs is needed. The information could then help with investigating strategies to inhibit the interactions. As an example, X-ray crystallography could be utilized to solve the SSL-MMP-complex structures. By obtaining the crystal structures it would be possible to identify the key amino acids contributing to the binding interface between the SSLs and the MMPs. However, MMPs can sometimes be difficult to work with because they need to be converted from the proenzyme to an active enzyme so that the active enzyme can be studied. Thus, scFv-93s binding ability could also be useful in crystallography studies. By solving the scFv-93-SSL1-complex structure, the binding site on SSL1 would be revealed and it would give information about how the SSL is inhibited.

6.6 The possibility to produce cross-reactive scFvs against the SSL proteins

The phage immunoassay results for the phage stocks selected to bind to SSL5 also showed slight binding against SSL1 (Figure 5). This can be observed after the second and third panning round, where the rate of SSL1 binding was 6.8 and 10.9% respectively. It is not a strong trend but could be an indication of something. The same effect could not be observed for the two other selections. The alignment of the SSL amino acid sequences demonstrates that SSL1 and SSL5 are more closely related compared to SSL10 (Supplemental figure 6). Because of this the two proteins might have some similar structural motifs that some scFvs are able to bind equally. The

reason why no binding to SSL5 can be seen in the SSL1 selection, could be because of the quicker enrichment towards the specific target (Table 3), not allowing for the collection of less specific binders (e.g. towards SSL5).

Depending on what the goal is, the cross-reactivity of scFvs can be either unwanted or even beneficial. If the effect of a scFv needs to be very specific, the cross-reactivity is an unfavorable trait, because it might divert the molecule from performing the wanted function. However, in other cases it could be a very beneficial trait. The SSL proteins are produced by *S. aureus* and they all support the bacteria in producing a successful infection. Being able to cross-neutralize several of the immune evasion molecules with one molecule would be a more powerful treatment. As an example, Rouha et al. (2015) were able to generate a human monoclonal antibody (mAb) that binds and neutralizes five *S. aureus* toxic molecules (a α-hemolysin and four bi-component leukocidins). They succeeded with this despite the molecules having quite a low overall amino acid sequence identity of ~26%. Another example of neutralizing bacterial toxins with cross-binding antibodies was accomplished by Garcia-Rodriguez et al. (2010). They identified two human mAbs that bound to two and four serotypes of botulinum neurotoxin, respectively. The amino acid sequence homology of these toxins ranged between 34-64%.

A Clustal2.1 multiple sequence alignment of the three SSLs reveals that the SSL1 and SSL5 share a sequence identity of 47.1% and in comparison, SSL10 shares only a ~34% identity with both SSL1 and SSL5 (Supplemental figure 6). This suggests that there might be a chance of generating cross-reactive antibodies towards SSL proteins as well. The best targets for a cross-neutralizing antibody would probably be SSLs that share an epitope that is conserved between the proteins and important for the proteins' function. SSL1 and SSL5 for instance both target MMPs and might have some conserved regions that are important for this activity. Therefore, the production of a cross-reactive antibody against SSL1 and SSL5 could be a future challenge.

7 Conclusions

S. aureus is one of the leading causes of infectious disease resulting in serious consequences (Lakhundi et al., 2018). Its remarkable ability to acquire resistance against multiple different antibiotics has driven research to find alternative ways of treating the bacterial infections. The immune evasion proteins that the bacteria secrete to ensure a successful infection can be a key factor in combating the bacteria. The goal in this thesis was to investigate how SSL proteins could be targeted for treatment and hopefully provide some novel information that could be useful in future research.

In this thesis, using two scFv phage display libraries we generated scFv antibody fragments that bind to three *S. aureus* immune evasion proteins: SSL1, SSL5 and SSL10. After performing multiple functional analyses on the scFvs we found that one scFv was successful at preventing SSL1 from inhibiting the MMP9 enzymatic activity. In a fluorogenic peptide MMP activity assay this was achieved at quite a low concentration of the scFv, 0.033 μM, which inhibited over 90% of 0.019 μM SSL1. This indicates that it is possible to produce neutralizing antibodies against the SSL proteins. Especially the potential of generating a cross-reactive antibody could be an effective way to disarm the bacteria from the molecules it uses to hide from the immune system.

8 Acknowledgements

This project was a collaboration between three labs, so naturally there are a lot of people I am extremely thankful for. First of all, I would like to sincerely thank Outi Salo-Ahen for giving me the opportunity to participate in this project. As well as Ida Alanko who I worked very closely with, and who supervised and encouraged me throughout this whole project. I would like to express my gratitude to Urpo Lamminmäki for letting me perform the phage display experiments in the Biotechnology department of University of Turku and Eeva-Christine Brockmann for the invaluable guidance and knowledge of the phage display technique. I am also very grateful that Jos van Strijp granted me the opportunity to carry out the functional analyses of our scFvs at the Medical Microbiology department at UMC Utrecht, which was a remarkably inspiring and rewarding experience. I am thankful for all the dedication and expertise that Carla de Haas and Lisette Scheepmaker provided while supervising my work in Utrecht. Finally, I would like to express how fortunate I have been when getting to know all the talented students in the labs, who became great friends and were a big support for me.

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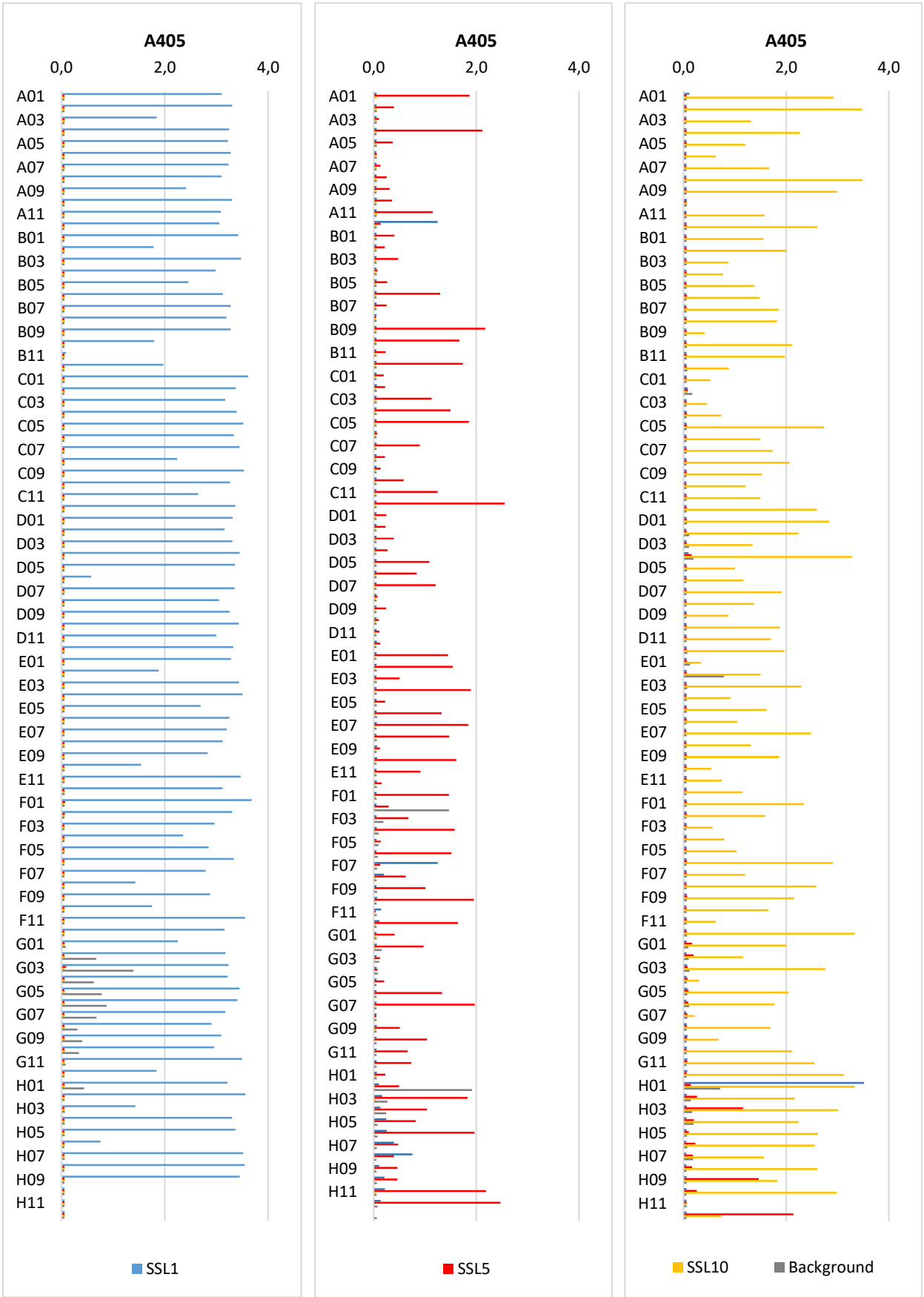
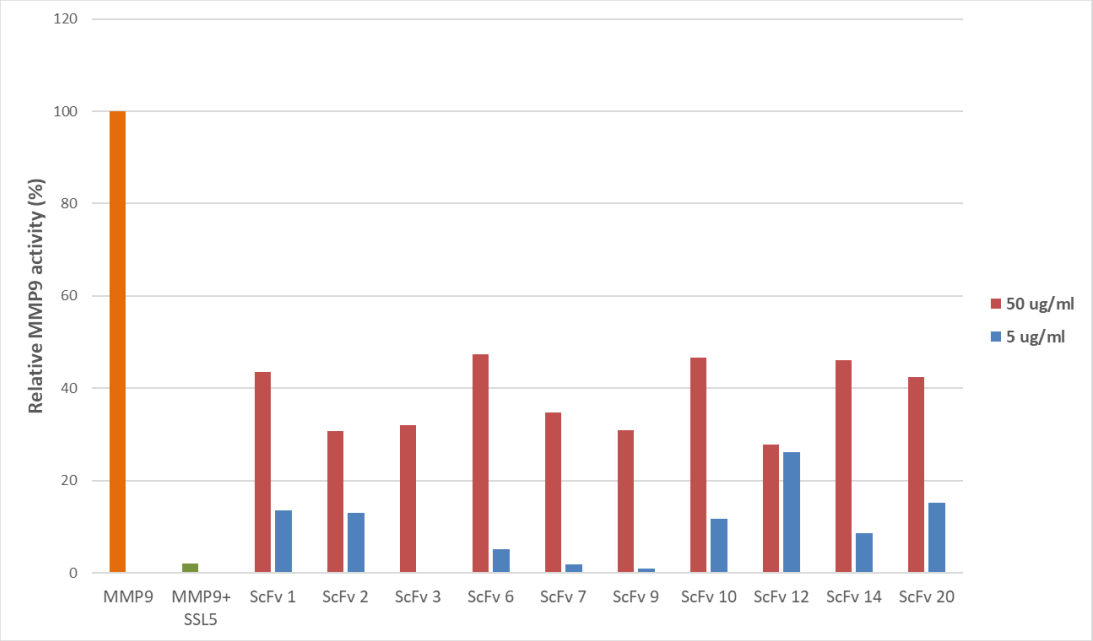
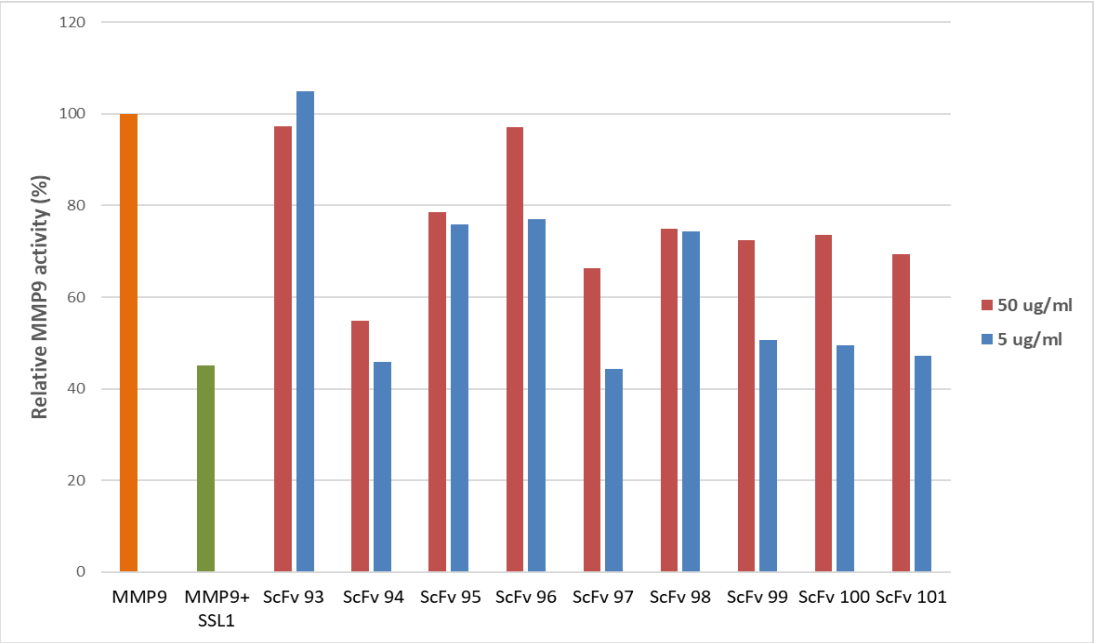
10 Supplemental results



Supplemental figure 1. Binding of individual clones of scFv-presenting phages proteins to SSL1, SSL5 and SSL10. The binding of the phages was measured against SSL1 (blue), SSL5 (red), SSL10 (yellow) and a control sample without any antigen (grey). The binding of individual clones of phages that were produced against (A) SSL1, (B) SSL5 and (C) SSL10 were measured in a scFv-ALP ELISA screening assay.



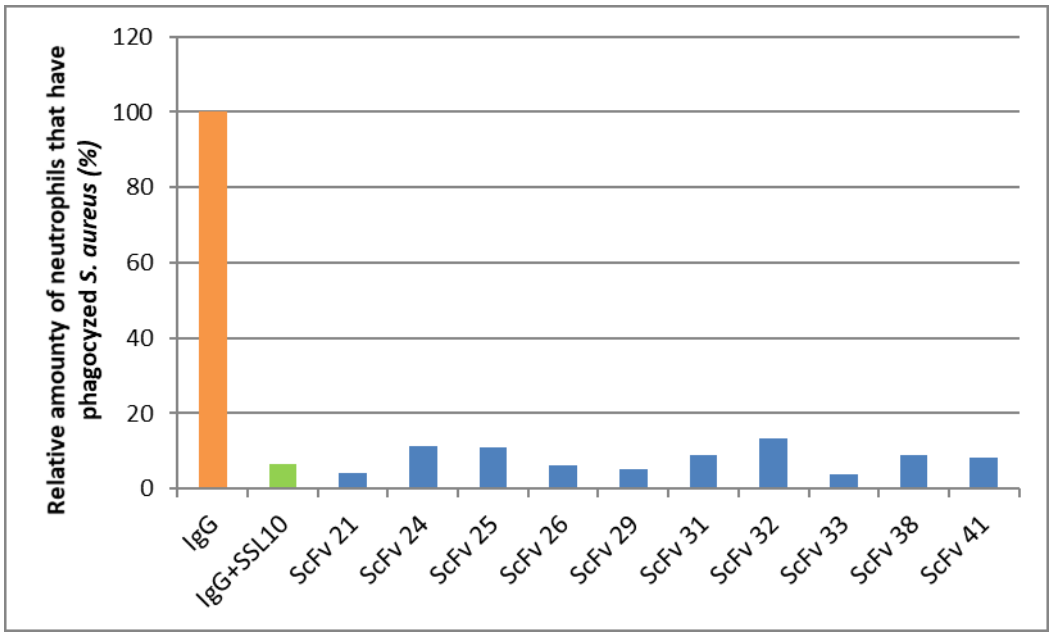
Supplemental figure 2. The recovery of SSL5-inhibited MMP9 activity by SSL5-binding scFvs. The scFvs recovering effect was tested at 50 µg/ml (red) and 5 µg/ml (blue). The SSL5 (0.125 µg/ml) inhibited MMP9 is shown in green and the uninhibited MMP9 activity (orange) was set to 100% and the activity adjusted in relation to this one.



Supplemental figure 3. The recovering effect of SSL1-binding scFvs on MMP9 activity being inhibited by SSL5. The scFvs recovering effect was tested at 50 µg/ml (red) and 5 µg/ml (blue). The SSL1 (0.5 µg/ml) inhibited MMP9 is shown in green and the uninhibited MMP9 activity (orange) was set to 100% and the activity adjusted in relation to this one.

ScFvs had no effect on SSL10-inhibited opsonization of S. aureus

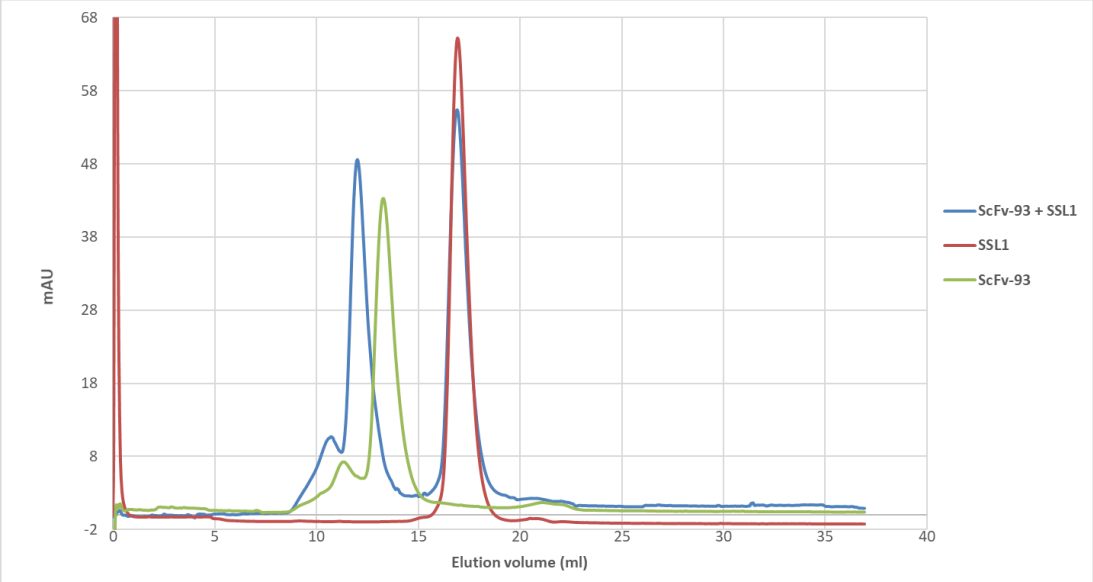
An assay where fluorescent *S. aureus* was used to measure the amount of neutrophils that had phagocytized bacteria. In order for the neutrophils to recognize and phagocytize the bacteria they have to be opsonized with IgG1. When the neutrophils have phagocytized the bacteria flow cytometry can be used to measure the amount of fluorescent neutrophils. However, SSL10 will inhibit the opsonization, if it is incubated with IgG1 before the reaction. Finally, by adding the SSL10-binding scFvs, their ability to recover the SSL10-inhibited opsonization could be measured. Supplemental figure 4 demonstrates that the tested scFvs showed no effect.



Supplemental figure 4. The relative amount of neutrophils that have phagocytized IgG1 opsonized *S. aureus*. The neutrophils full capacity to phagocytize the opsonized bacteria was measured (orange) and the rest of the signals were adjusted in relation to this. Opsonization was inhibited with 2.5 ug/ml SSL10 (green) and the effect of adding 25 ug/ml scFvs was observed (blue). Experiment performed by MSc Ida Alanko.

Gel filtration chromatography confirmed that scFv-93 binds to SSL1

Gel filtration chromatography was used to establish whether scFv-93 binds to SSL1. In this technique proteins are separated according to their size by eluting the proteins through a column containing a porous matrix (Hagel, 2001). The smaller molecules will, for steric reasons, travel slower through the matrix allowing larger molecules to be eluted quicker. Therefore, SSL1 that has the smallest size gives rise to an elution peak around 17 ml, whereas scFv-93 alone produces a peak around 13 ml (Supplemental figure 5). When scFv-93 is filtrated together with SSL1, two peaks are generated. One peak around 12 ml that illustrates that the two molecules produce a complex together as well as a peak that account for unbound SSL1.



Supplemental figure 5. Gel filtration chromatography of the scFv-93 together and as a complex. Experiment performed by Assistant Professor Dr. Carla J. C. de Haas.

A

```
SSL10       -------------------------KQNQ-------KSVNKHDKEALYRYYTGKTMEMK  27
SSL1        MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKKAEVK-----QQSESELKHYYNKPILERK  55
SSL5        MRGSHHHHHHGMASMTGGQQMGRDLYDDDDKSEHKAKYENVTKDIFDLRDYYSGASKELK  60
                                  .:::            :.   *  **.    * *

SSL10       NISALKH---GKNNLRFKFRGIKIQVLLPGNDKSKFQQRSYEGLDVFFVQEKRDKHDIFY  84
SSL1        NVTGFKYTDEGKHYLEVTVGQQHSRITLLGSDKDKFKDGENSNIDVFILREGDSRQATNY  115
SSL5        NVTGYRYSKGGKHYLIFDKNRKFTRVQIFGKDIERFKARKNPGLDIFVVKEAENRNGTVF  120
            *         **  * .       :: : *.* .:*:  .  .:*:*.::*  .::   :

SSL10       TVGGVIQNNKTS--GVVSAPILNISKEKGEDAFVKGYPYYIKKEKITLKELDYKLRKHLI  142
SSL1        SIGGVTKSNSVQYIDYINTPILEIKKDNEDVLK---DFYYISKEDISLKELDYRLRERAI  172
SSL5        SYGGVTKKNQDAYYDYINAPRFQIKRDEGDGIATYGRVHYIYKEEISLKELDFKLRQYLI  180
            : *** :.*.    . :.:* ::*.::: :        :** **.*:*****::**:  *

SSL10       EKYGLYKTISKDGRVKISLKDGSFYNLDLRSKLKFKYMGEVIESKQIKDIEVNLK       197
SSL1        KQHGLYSNGLKQGQITITMNDGTTHTIDLSQKLEKERMGESIDGTKINKILVEMK       227
SSL5        QNFDLYKKFPKDSKIKVIMKDGGYYTFELNKKLQTNRMSDVIDGRNIEKIEANIR       235
            ::..**..  *:.::.: ::**  :.::* .**: : *.: *:. :*:.* .:::
```

B

	SSL10	SSL1	SSL5
SSL10	100.00	34.21	34.01
SSL1		100.00	47.14
SSL5			100.00

Supplemental figure 6. Protein sequence alignment of SSL1, SSL5 and SSL10 was performed with Clustal2.1. A) A multiple sequence alignment of the SSL proteins. The asterisk indicates that the amino acid is fully conserved, the colon indicates conservation with strongly similar amino acids and the period indicates conservation with less similar amino acids. B) Percent identity matrix of SSL proteins.

11 Recipes

TSA/BSA:

50 mM Tris

150 mM NaCl

1% BSA

0.02% NaN3

pH 7.5

TBT-0.05:

50 mM Tris

150 mM NaCl

1% BSA

0.05% Tween

pH 7.5

SB medium:

30 g/l Tryptone

20 g/l Yeast Extract

10 g/l MOPS

pH 7.0

LA-Plates:

10 g/l Tryptone

5 g/l Yeast Extract

15 g/ Agar

0.5% Glucose

Phage quantification assay probe mix:

10 nM Eu-oligo

5 nM antenna-oligo

25 mM Tris-Cl

900 mM NaCl

0.1% Tween-40

0.05% NaN_3

30 µM DTPA

pH 7.5

S.O.C. medium:

2 % Tryptone

0.5% Yeast Extract

10 nM MaCl

2.5 mM KCl

10 mM $MgCl_2$

10 mM MgSO4

20 mM Glucose

10x lysis buffer:

10 mg/ml Lysozyme (L6876-10G, Sigma-Aldrich, USA)

10 mM $MgCl_2$

25 U/ml Benzonase (1.01654.0001, Merck, Germany)

pNPP buffer:

500 mM Tris

200 mM NaCl

10 mM $MgCl_2$

pH 9.0

Fluorogenic peptide assay buffer:

50 mM Tris

10 mM CaCl2,

150mM NaCl

0.05% Brij-35

pH 7.5

Trypsin inhibition cocktail:

10 mM Pefabloc (Sigma-Aldrich, USA)

3,5 mg/ml STBI

100 mg/ml Aprotinin (Sigma-Aldrich, USA)

12 Svensk sammanfattning

Isolation och funktionell analys av fagdisplay-producerade scFv-antikroppar som binder *S. aureus* SSL proteiner

12.1 Introduktion

Staphylococcus aureus är en gram positiv bakterie som är ofta förekommande hos människor, främst på huden och i mukosala membran. Bakterien agerar ofta som kommensal hos människan men den räknas ändå inte till människans normalflora eftersom den även kan fungera som patogen och i värsta fall vara dödlig. *S. aureus* har en förmåga att utveckla antibiotika resistenta stammar som kallas meticillinresistent *S. aureus* (MRSA) (Chambers et al., 2009). I dagens läge är MRSA en av de främsta bakterierna som orsakar antibiotikaresistens i Europa och är därför en stor börda för hälsovårdssystemet. År 2015 orsakade MRSA uppskattningsvis 150 000 infektioner och 7000 dödsfall i Europa (Quattrocchi et al., 2019).

En annan viktig orsak till *S. aureus* är en så framgångsrik patogen är dess förmåga att undgå värdens immunförsvar. Idag känner man till mer än 35 olika proteiner som *S. aureus* använder för att undkomma immunförsvaret, vilket är mer än vad man har funnit i någon annan bakterie (Koymans et al., 2017). Det är främst det medfödda immunförsvaret som är det första försvarssteget mot infektioner är målet för dessa proteiner.

I detta projekt har vi fokuserat på tre proteiner som *S. aureus* använder för att undvika värdens immunförsvar: stafylokockssuperantigenlika (SSL) proteinerna 1, 5 och 10. Både SSL1 och SSL5 binder och inhiberar den enzymatiska funktionen av åtta matrixmetalloproteinaser (MMP) (Koymans et al., 2016). Ursprungligen beskrevs MMP proteiner som proteinaser som byter ned extracellulärt matrix, men senare har det upptäckts att de även har en viktig roll i immunförsvaret och inflammation, speciellt vid bakterie infektioner, sår läkning och cancercellinvasion (Koymans et al., 2016). SSL5 proteinet kan ytterligare motverka neutrofilers migration till infektions platsen genom att binda till P-selektin glykoprotein ligand-1 (PSGL-1). Dessa ligander uttrycks på neutrofilers yta och växelverkar med P-selektin som finns på endotelceller, som täcker blodkärlens yta (Bestebroer et al., 2014). Denna interaktion möjliggör neutrofilers rörelse i riktningen av infektionen.

SSL10 proteinet skiljer sig mer från de två andra SSL proteinerna vi undersökte, men det deltar ändå i försvagandet av värdens immunförsvar. SSL10 påverkar immunförsvaret huvudsakligen genom att binda till IgG och blockera dess bindning till C1q ocg FcγR. Detta hindrar C1q från att initiera komplementsystemet och FcγR från att stimulera monocyters och neutrofilers fagocytos av patogener (Itoh et al., 2009; Patel et al., 2010).

Tillsammans kan SSL1, SSL5 och SSL10 motverka värdens immunförsvar på många plan, vilket gör en *S. aureus* infektion svårare att bekämpa. På grund av *S. aureus* förmåga att utveckla antibiotika resistens finns det ett behov av att utreda alternativa metoder för att bekämpa bakterieinfektionerna. Vi valde att utforska möjligheten att producera scFv-antikroppar (enkelkedjade variabla fragment, eng. single-chain variable fragment) som kunde inhibera SSL1, SSL5 och SSL10 proteinernas immunhämmande funktioner.

12.2 Målsättningar

Målet i detta projekt var att producera antivirulensmedel som binder till och hindrar SSL proteiner från att försvaga immunförsvaret. Dessa skulle kunna användas tillsammans med antibiotika vid allvarliga *S. aureus* infektioner. Målsättningen med denna studie var att:

- Producera bindare till *S. aureus* proteinerna SSL1, SSL5 och SSL10 med hjälp av fagdisplaytekniken.
- Testa hur effektivt de isolerade bindarna binder till de tre SSL proteinerna med hjälp av fluorescensbaserad immunanalys.
- Välja ut och producera de scFv-antikroppar som visat den bästa bindningsförmågan i fluorescensbaserad immunanalys, genom att klona fusionsproteinuttryckande genen från fagerna till Mach-T1 celler.
- Testa om scFv-antikropparna klarar av att hindra SSL1 och SSL5 från att inhibera MMP9 eller om de klarar av att hindra SSL10 från att binda DNA.

12.3 Metoder

Fagdisplay tekniken användes för att producera scFv-antikroppar som binder specifikt till SSL1, SSL5 och SSL10 proteinet. Dessa proteiner immobiliserades på magnetiska

streptavidinpärlor varefter ett bibliotek av bakteriofager som uttryckte olika varianter av scFv-antikroppar fick binda till proteinerna. De fager som band till SSL proteinerna amplifierades i XL-1 Blue *Escherichia coli* bakterier så att en ny samling av fager producerades. Sedan upprepades denna procedur, som även kallas panning, ytterligare två gånger för att sålla fram de bästa bindarna.

Fluorescensbaserad immunanalys användes för att mäta vilka scFv-antikroppar som band bäst till mål proteinerna SSL1, SSL5 och SSL10. Därefter klonades generna för de bäst bindande scFv-antikropparna som ett fusionsprotein med alkaliskt fosfatas (ALP, eng. alkaline phosphatase) in i Mach1-T1 bakterier. Dessa bakterier användes till att producera scFv-ALP proteinerna.

ScFv-antikropparna kunde sedan testas i en rad funktionella analyser för att ta reda på om de hade en inhiberande effekt på SSL proteinernas funktion. En fluorogenisk peptidanalys metod användes för att mäta om scFv-antikropparna kunde inverka i SSL1 eller SSL5 proteinernas kapacitet att inhibera MMP9. ScFv-antikropparnas förmåga att hindra SSL10 från att binda till DNA undersöktes med gel-retardationsanalys.

12.4 Resultat

De scFv-antikroppar som producerats till att binda SSL1 och SSL5 testades med en fluorogenisk peptidanalys. MMP9 enzymets aktivitet kunde mätas med hjälp av en peptid, som avger en fluorescerande signal när det blir kluvet av enzymet. SSL1 och SSL5 har förmågan att blockera enzymets aktivitet vilket minskar den fluorescenta signalen. Av de SSL5 bindande scFv-antikropparna gav sju olika kloner mellan 20 och 50 % återhämtning av MMP9 enzymets aktivitet (Figur 14). Detta uppnåddes dock endast vid en 50 µg/ml koncentration och när koncentrationen minskade till 10 µg/ml eller lägre tappades effekten helt.

De SSL1 bindande scFv-antikropparna hade en starkare inverkan på SSL1 proteinets förmåga att blockera MMP9. Fyra stycken scFv-antikroppars effekt mättes i olika koncentrationer med ett intervall från 50 µg/ml till 0,16 µg/ml (Figur 15). Den högsta koncentrationen gav en återhämtning mellan 75 och 90 %. För scFv-95, 96 och 98 sjönk effekten i förhållande till de lägre koncentrationerna. ScFv-93 däremot hade en över 90 % återhämtande effekt ned till en 2,5 µg/ml koncentration.

ScFv-93 undersöktes vidare i en kollagen spjälkningsanalys. Kollagen inkuberades tillsammans med MMP9 enzymet, med eller utan SSL1 och scFv-93 i olika koncentrationer. Efter en övernatt inkubation separerades proteinerna i en natriumdodecylsulfat-polyakrylamidgelelektrofores (eng. sodium sulfate polyacrylamide gel electrophoresis, SDS-PAGE). Detta möjliggjorde att kollagenets nedspjälkningsgrad, som orsakats av MMP9, kunde visualiseras. En koncentration av 30 µg/ml och 15 µg/ml scFv-93 kunde delvis hindra SSL1 från att blockera nedbrytningen av kollagenet (Figur 16).

SSL10 proteinets förmåga att binda till DNA kunde åskådliggöras i en gel-retardationsanalys. De SSL10 bindande scFv-antikroppar hade ingen synlig effekt på denna bindning (Figur 18).

12.5 Diskussion

I detta projekt lyckades vi sålla fram scFv-antikroppar som binder till SSL1, SSL5 och SSL10. I de funktionella analyserna visade sig en SSL1-bindande scFv-antikropp ha en önskad effekt. ScFv-93 inhiberade SSL1 till 90 % vid en koncentration av 2,5 µg/ml (0,033 µM). SSL1 koncentrationen som användes var 0,5 µg/ml (0,019 µM), vilket betyder att ett 1,7-faldigt molärt överskott av scFv-93 lyckades inhibera SSL1 i hög grad. Eftersom ett scFv-antikropp endast kan binda en molekyl åt gången skulle den maximala effekten vara att ett 1:1 molarigt förhållande skulle leda till en fullständig inhibition. Dessa resultat tillsammans med kollagen spjälkningsanalysen tyder på att scFv-93 har en återhämtande effekt på MMP9-enzymet när det inhiberas av SSL1.

Ingen av de SSL5 bindande scFv-antikropparna uppnådde en lika stark hämmande effekt på SSL5 proteinets förmåga att inhibera MMP9. ScFv-antikropparna lyckades inte heller förhindra SSL10 från att binda till DNA eller förhindra SSL10 från att inhibera fagocytos av *S. aureus*. Det är möjligt att scFv-antikropparna kan binda till andra delar av SSL proteinerna. Därför kunde deras effekt undersökas ytterligare för att ta reda på om några andra av SSL proteinernas funktioner kunde inhiberas.

SSL1 proteinet har även visats kunna inhibera flera andra MMP proteiner (MMP1, 2, 7-9, 12-14) (Koymans et al., 2016). Det skulle vara intressant att undersöka ifall scFv-93 även skulle kunna hindra SSL1 från att inhibera de andra MMP-proteinerna. Detta skulle tillsammans med kristallografiska analyser av ett scFv-93-SSL1-komplex

kunde samtidigt ge mera information om växelverkan mellan SSL1 proteinet och MMP enzymen det binder.

Detta är ett steg närmare en behandlingsmetod som kunde användas till att bekämpa *S. aureus* bakterieinfektioner. Genom att inhibera bakteriens immunundvikningsproteiner kan värdens immunförsvar åstadkomma en starkare immunrespons vilket leder till att en infektion elimineras snabbare.