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Interaction between Extracellular adherence protein (Eap) from *Staphylococcus aureus* and the human host

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La verdadera felicidad no consiste en tener todo cuanto se desea, sino en desear cosas que no se tienen y en luchar por conseguirlas.

Julio Antonio Mella

For my family

Abstract

Staphylococcus aureus is one of the most common agents causing bacterial infections in humans and animals. The emergence of extended antibiotic resistance among *S. aureus* strains as a worldwide epidemic has driven the development of alternative strategies to combat this microorganism.

This thesis is based on the interaction between Extracellular Adherence Protein (Eap) from *S. aureus* and the human host. Eap is an extracellular protein capable of binding to several plasma proteins including fibronectin, fibrinogen and prothrombin. Adherence and internalization into host cells are key steps during the infectious process; adherence for successful colonization and internalization to shield the bacteria from host defense and antibiotic treatment. Using a mutant strain, Newman AH12 lacking the *eap* gene we were able to demonstrate a clear role for Eap in adherence and internalization. Strain Newman (wild type strain) could adhere to and become internalized better by eukaryotic cells than the isogenic mutant and externally added Eap enhanced adherence and internalization of strain Newman, Newman AH12, and clinical strains. Antibodies against Eap were able to block the adherence and internalization process in strain Newman.

In the second part of this thesis we confirm the role of Eap as an immunomodulating protein. We could demonstrate that Eap inhibits the binding of neutrophils to the endothelium under static and dynamic flow conditions and also inhibits transendothelial migration of neutrophils in an in vitro model. We could also show that Eap blocked to the same extent as human ICAM-1 antibodies. This data together with previous reports suggest that Eap also in this setting elicits its effect mainly by binding to ICAM-1. On the other hand, we could also demonstrate a direct effect of Eap on peripheral blood mononuclear cells (PBMCs), which was concentration dependent. At low concentrations, Eap elicited a stimulatory effect on PBMC and at high concentrations it had an inhibitory effect through induction of apoptosis in T and B cells.

Protective cellular immunity is the main mechanisms of defense used by the host to eliminate *S. aureus* infection. The presence of Eap during a *S. aureus* infection will have a major impact on this system by virtue of (i) Eap mediated adherence and internalization into host cells, (ii) inhibition of neutrophils migration to the site of infection (iii) inhibition of T and B cell proliferation. Hence, these studies imply that intervention directed against Eap could be a novel approach to prevent or improve therapy of Staphylococcal infections.

List of publications

This thesis is based on the following papers:

Paper I
Muzaffar Hussain, **Axana Haggar**, Christine Heilmann, Georg Peters, Jan-Ingmar Flock and Mathias Herrmann.
Insertional Inactivation of *eap* in *Staphylococcus aureus* Strain Newman Confers Reduced Staphylococcal Binding to Fibroblasts.
Infection and Immunity 2002 June, vol.**70** (6), p. 2933-2940

Paper II
Axana Haggar, Muzaffar Hussain, Helena.Lönnies, Mathias Herrmann, Anna Norrby-Teglund and Jan-Ingmar Flock.
Extracellular Adherence Protein from *Staphylococcus aureus* Enhances Internalization into Eukaryotic Cells.
Infection and Immunity 2003 May; vol. **71** (5), p. 2310-2317

Paper III
Axana Haggar, Cecilia Ehrnfelt, Jan Holgersson and Jan-Ingmar Flock**.**
Extracellular adherence protein from *Staphylococcus aureus* Inhibits Neutrophil Binding to Endothelium Cells.
Infection and Immunity 2004 October; vol. **72** (10) p. 6164-6167.

Paper IV
Axana Haggar, Oonagh Shannon, Anna Norrby-Teglund and Jan-Ingmar Flock
Dual Effects of Extracellular Adherence Protein from *Staphylococcus aureus* on Peripheral Blood Mononuclear Cells.
The Journal of Infectious Diseases 2005 July, vol.**192**, p.210-7

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List of Abbreviations

Eap	Extracellular adherence protein
FnBPs	Fibronectin binding proteins
FgBP	Fibrinogen binding protein
ECMBP	Extracellular matrix binding protein
Fg	Fibrinogen
Clf	Clumping factor
Efb	Extracellular fibrinogen binding protein
Emp	Extracellular matrix protein-binding protein
InlA or B	Internalin A or Internalin B
Map	Major histocompatibility complex class II analogous protein
ICAM-1	Intracellular adhesion molecule
LFA-1	Lymphocyte function associated antigen 1
TNF-α	Tumor necrosis factor alpha
HAECs	Human aortic endothelial cells
PBMCs	Peripheral blood mononuclear cells
IgG	Immunoglobulin G
IVIG	Pool of human polyspecific IgG
TSST-1	Toxic shock syndrome toxin
Sak	Staphylokinase
APCs	Antigen presenting cells
Th-cell	T-helper cells
CHIPS	Chemotaxis inhibitory protein of Staphylococcus aureus
SCIN	Staphylococcal complement inhibitor
SSL-protein	Staphylococcal superantigen-like protein

Introduction

Staphylococcus aureus

Staphylococcus aureus is a gram-positive, nonmotile, catalase-positive coccus. It was Sir Alexander Ogston who first described staphylococcus and its role in the infectious diseases, more than 100 years ago (81). *S. aureus* is a common commensal of humans. It colonizes the nares, axillae, vagina, pharynx or damage skin surface and it can be present persistently (20%) or intermittently (60%). Twenty % of the population will never be carriers of the bacteria (91, 112). *S. aureus,* which can cause a wide range of diseases, is best known as a community pathogen owing to its ability to produce furuncles and infect soft tissue. More life threatening diseases caused by *S. aureus* are endocarditis, osteomyelitis and septic shock. Nosocomial infections caused by *S. aureus* have increased in the last decades. Methicillin resistant *S. aureus* (MRSA) strains, with resistance against most clinically useful antibiotics, commonly contribute to 10-60% of all strains of *S. aureus* isolated in hospitals. As a result of this, vancomycin is often the only effective antibiotic remaining. Already, clinical studies have shown the existence of MRSA strains with reduced sensitivity and even resistant to vancomycin, which almost returns us again to a pre-antibiotic era (18, 44, 45). The emergence of these extended antibiotic-resistant *S. aureus* strains as a worldwide epidemic has made it necessary to develop new strategies to understand the different mechanisms used by the bacteria at the different stages of the infectious process. This knowledge will provide us with the tools to prevent or at least improve the control of *S. aureus* infections.

Pathogenesis and Virulence factors

Since about 50% of humans are a natural reservoir for *S. aureus,* most of us live in harmony with the bacteria, until the balance between the host and the bacteria is taken out of equilibrium. The ability of *S. aureus* to establish a niche in the host is a crucial step in its pathogenesis. The capacity to infect and disseminate into the bloodstream and distant sites in the host is mostly dependent on mucosal barriers being breached or the host immunity being affected. Access and adherence to host tissue or implanted

materials is mediated by surface receptors or adhesins and involves host protein interactions. More specifically the interaction occurs between adhesins of the bacterial cell wall surface and target structures on the eukaryotic cells. In the case of implants or surgical intervention, plasma proteins are immediately deposited on the materials or where a thrombus has been formed. Host proteins act as bridging molecules in the adherence of *S. aureus* to protein coated surfaces. *S. aureus* is well equipped with many extracellular matrix binding proteins (ECMBPs) also known as adhesins, receptins (62) or Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (30). The term ECMBPs is going to be used in this thesis and some of these proteins will be mentioned in the next section.

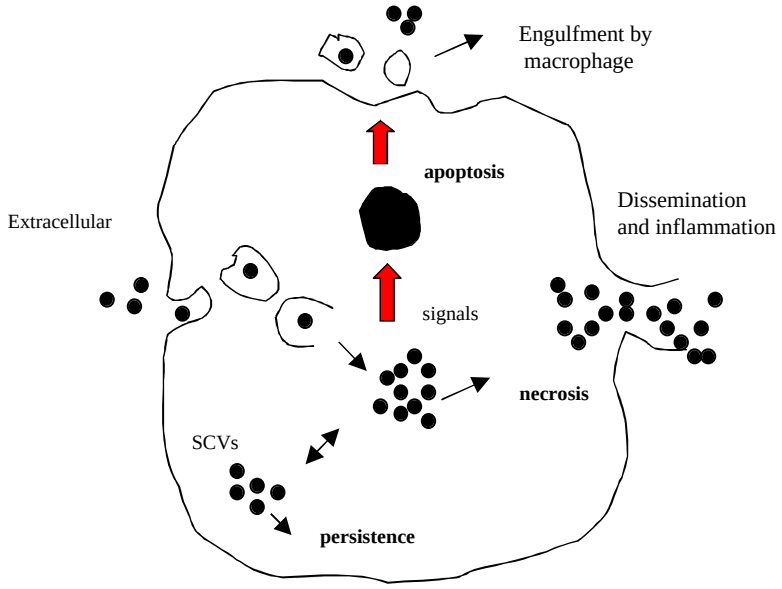

Figure 1. Internalization of *S. aureus* into eukaryotic cells. Once *S. aureus* has been internalized in the cytoplasm of the host, we envision three possible outcomes: (i) the induction of apoptosis, (ii) the formation of small colony variants, which leads to intracellular persistence (113), or (iii) lysis of the host cell, picture modified from Wesson et al. 1998.

Until recently *S. aureus* was regarded as a mainly extracellular bacteria, mostly because of the staining performed to identify the bacteria, one could see the bacteria like a bunch of grapes (from the greek staphyle) around the cells. The fact that *S. aureus* can be internalized into non-professional phagocitic cells is well documented (4, 56, 75). Internalization of the bacteria into non-professional phagocytic cells has been associated with (i) persistent and relapsing infections, due to their intracellular

location, shielding the bacteria from host defense and antibiotic treatment, (ii) apoptosis leading to engulfment by macrophages and (iii) necrosis leading to dissemination of the bacteria in the host as shown in Figure1 (118). Although most ECMBPs interact with specific host proteins, many have overlapping functions and are involved in the adherence and internalization of the bacteria into eukaryotic cells. This makes the study of these proteins very complex.

S. aureus produces other virulence factors, which are associated with more defined diseases/syndromes, such as superantigens. toxic shock syndrome toxin (TSST-1) is responsible for toxic shock syndrome associated with the use of superabsorbent tampons in the beginning of the 80ies. (SE) staphylococcal enterotoxins are responsible for food poisoning. In brief, superantigens elicit their function by bypassing the conventional antigen recognition by direct cross-linking MHC II on antigen presenting cells (APC) with T-cells receptors on T lymphocytes in an indiscriminate manner (48). This results in a massive immune response from the host. Other toxic proteins, which are well studied are the exfoliative toxins responsible for the scalded skin syndrome, now a days a very rare disease and mostly found in infants; staphylokinase (SAK) which activates plasminogen and thereby dissolves fibrinogen clots and interferes with the wound healing process; leukocidin which damage mammalian cell membrane. The list of virulence factors is long (48, 70).

More recent findings have shown that *S. aureus* also has other specific proteins that can have profound impact on the innate and adaptive immune system. The best example in this field would be protein A, which binds to the Fc portion of the antibody, in such a way that opsonization of bacteria is inhibited (95). SAK has also shown to have an influence on the innate immune response. α-Defensin are bactericidal peptides produced by human neutrophils. Binding of Sak to α-defensins abolished their bactericidal properties (10, 99). Other immunomodulating proteins will be mentioned briefly here and addressed further in this thesis, for example Extracellular fibrinogen binding protein (Efb), Chemotaxis Inhibitory Protein of *S. aureus* (CHIPS) and Staphylococcal Complement Inhibitor (SCIN) which have been shown to interfere with the complement system (21, 66, 67, 96, 98) and Extracellular adherence protein (Eap) which has been shown to interfere with transendothelial migration of neutrophils and T-cell proliferation (15, 68)(Paper III and IV).

Extracellular matrix binding proteins (ECMBPs)

Access and adherence to host tissues or implanted materials is mediated mainly by ECMBPs from *S. aureus*. ECMBPs are attached to the bacterial cell wall surface and interact with target structures on the eukaryotic cells. Many staphylococcal surface proteins have certain structural features in common. These features include a secretory signal sequence at the N-terminal, positively charged amino acids that extend into the cytoplasm, a hydrophobic membrane spanning domain, and a cell wall anchoring region. Most ECMBPs are attached to the staphylococcal cell wall by a common mechanism, the LPXTG sequence (71). *S. aureus* produces a number of ECMBPs with high affinity and specificity for the corresponding matrix protein among others, fibronectin binding proteins (FnBPs) (28, 105), a collagen binding protein (88), fibrinogen binding proteins (FgBP) (6, 72), a vitronectin binding protein (89) and an elastin binding protein (87). In addition, four extracellular proteins, lacking the LPXTG motif, with ability to bind to fibrinogen are produced by *S. aureus*: coagulase, Efb, Extracellular matrix protein-binding protein (Emp) (50) and Eap. These proteins have been recently grouped and named, Secretable Expanded Repertoire Adhesive Molecules (SERAM) (17). The common denominator is their role in the association with endovascular disease produced by *S. aureus*. Most of these ECMBPs have specific biological functions and are proposed to contribute to successful colonization and persistence at various sites within the host.

Fibronectin binding proteins

Fibronectin is an extracellular matrix protein responsible for linking the components of the matrix both to one another and to the surface of cells, principally as an adhesive protein of connective tissues. *S. aureus* produces two FnBPs, fibronectin binding proteins A (FnBPA) and B (FnBPB). Either one or both of the FnBPs are expressed in *S. aureus*, with a prevalence of 100% for at least one *fnb* gene *(90, 92)*. FnBPA has been linked to adhesion and invasion of mammalian cells (epithelial, endothelial and fibroblast cells). The binding and internalization is believed to depend on fibronectin bridging between FnBPs on *S. aureus* and the host fibronectin receptor integrin $(\alpha_5)\beta_1$ which can lead to actin-mediated phagocytosis of the adherent bacteria (106). Besides

its ability to bind fibronectin, FnBPs have also been shown to bind to fibrinogen (115). More recent studies have shown a biological difference between the two FnBPs. It was shown that FnBPs could mediate adherence of *S. aureus* to platelets through bridging by either fibronectin or fibrinogen. On the other hand, aggregation of platelets was mainly induced by FnBPA and not by FnBPB (41). Furthermore in an experimental model of septic arthritis FnBPs seemed to have a distinct role in systemic inflammation during staphylococcal infection (85).

Fibrinogen binding proteins

Fibrinogen (Fg) is a high molecular weight protein produced in the liver and is one of the essential factors in the coagulation process. Thrombin is an enzyme with proteolytic capacity and acts on fibrinogen by removing four low molecular weight peptides from each molecule of fibrinogen, forming monomeric fibrin molecules. Fibrin monomer molecules polymerize into long fibrin threads that will form the blood clot. *S. aureus* produces more than half a dozen different fibrinogen binding proteins indicating that the binding to fibrinogen is important for *S. aureus* pathogenesis as described below.

Clumping factor,Clf

Binding of *S. aureus* to fibrinogen is mainly due to the cell wall associated protein clumping factor (Clf-A and B). The association of Clf with fibrinogen gives the characteristic phenotype of the ”clumping of the bacteria” in the presence of fibrinogen where the name originates. Clf- A and Clf- B bind to fibrinogen at different sites, Clf-A binds to the γ -chain of fibrinogen and Clf-B to Aα-and Bβ- chains of fibrinogen. Clf-A is present at all stages of the bacterial growth, in contrast to Clf-B, which is only present during the early exponential phase of the bacterial growth (25, 72). Clf´s biological role was demonstrated when a clumping factor deficient mutant was shown to have up to 100 times lower affinity for fibrinogen and produced 50% less endocarditis in rats compared to the native strain (109). More recently Clfs has been shown to contribute to septic arthritis produce by *S. aureus* infection (85).

Extracellular fibrinogen binding protein, Efb

Efb is an extracellular protein with the molecular weight of 15.6 kDa. Efb is present in 68 to 100% of *S. aureus* isolates, but in none of the coagulase negative species (8, 92). Efb binds to the α-chain of fibrinogen and does not participate in bacterial adherence to fibrinogen. The binding of Efb to fibrinogen is divalent, either due to two binding sites on Efb to fibrinogen or due to formation of dimers. This divalent binding leads to precipitation of Efb-fibrinogen complex when the proteins are added to each other at the same concentrations (84). On the contrary, Lee et al. claim that only the N-terminal region binds to fibrinogen and that no binding is seen in the C-terminal (66). Efb has been shown to be involved in the delay of the wound healing process due to its specific inhibition of platelet aggregation; platelet inhibition by Efb was seen both *in vitro* and *in vivo*. Antibodies against Efb were able to neutralize Efb in vitro and by this block the biological effects of Efb (83, 103, 104). In addition, it has been shown that Efb binds to the C3b fragment of C3 and inhibits both the classical and alternative pathways of complement activation (67).

Extracellular Adherence protein, Eap

Extracellular Adherence Protein, **Eap** is an exiting protein produced by *S. aureus* and has been the subject of investigation of different groups around the world for more than a decade. Studies have spanned from basic phenotypic observations, to more precise biological functions, up to the crystal structure of the Eap–domains. Three different groups have worked independently with this protein and build up the knowledge we have of Eap.

Youssif et al. published in 1991 the finding of two novel highly cationic proteins, neutral phosphatase (Nptase) and a 70-kD protein. The latter of which was named p70 and had high affinity for the glomerular basement membrane of rats (119). A few years later (1995) Jahreis et al. continued their work by showing that p70 could induce immunogobulin synthesis in peripheral blood mononuclear cells (PBMC), proliferation of T-cells and induce stimulation of resting B-cells in an non-antigen-specific manner. Jahreis et al. (2000) could also see that the presence of p70, induced IL-4 production in PBMC in *vitro,* which could have an impact on the T-cells

response, switching it from cellular (Th1) to humoral (Th2) and thus impairing the ability to clear *S. aureus* infections (54, 55).

The second group started their studies in 1992, when Bodén et al. purified three different fibrinogen- binding proteins. An 87 kDa fibrinogen binding coagulase and a 60kDa fibrinogen and prothrombin- binding protein. The first protein was produced mainly during the exponential growth phase and the second one during the post-exponential growth phase. In addition, a 19 kDa fibrinogen-binding protein was constitutively produced (later named Efb) (6). Palma et al. (1999) characterized the 60kDa protein described by Bodén et al. and found that this protein was mostly extracellular (70%) and to a lesser extent bound to the bacterial surface. It could bind at least seven plasma proteins, including fibrinogen, fibronectin and protrombin and the maximal production of the protein was at late exponential phase. This protein caused agglutination of the bacteria due to its ability to rebind to the surface of *S. aureus* and because of the strong tendency of the protein to form multimeric aggregates. Exogenously added protein significantly enhanced the adherence of *S. aureus* to fibroblasts and epithelial cells. Based on the broad binding activity and its role in the adherence enhancement to eukaryotic cells, Palma named the protein Eap, extracellular adherence protein (82).

The third group started their studies in 1993, when McGavin et al. was attempted to find a receptor for bone sialoprotein (BSP). They purified an extracellular matrix-binding protein with broad specificity. This 72- kDa protein bound, not only to BSP, but also fibronectin, fibrinogen, vitronectin, thrombospondin and even collagen (73). Jönsson et al. continued this work by cloning and sequencing the gene for this broad spectrum matrix-binding protein. This protein consisted principally of six repeated domains of 110 residues. Each of the repeats contain a sub-domain of 31 residues that had sequence homology with a segment in the peptide binding groove of the β- chain of the major histocompatibility complex (MHC) class II from eukaryotic cells as shown in Figure 2. This protein was named Map for Major Histocompatibility Complex Class II analogous Protein (58).

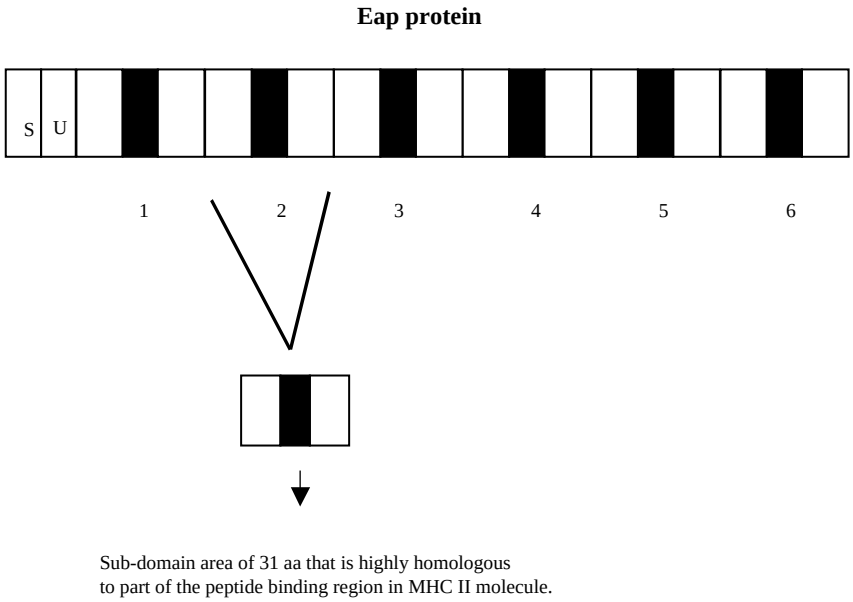

Figure 2. Structure of the Eap protein. The protein consists of a signal sequence (S) of 30 amino acids followed by a unique sequence (U) of 19 amino acids and six repeated domains (1-6) of approximately 110 amino acids. Within each domain there is a subdomain of 31 amino acids that is highly homologous to part of the peptide binding region in MHC class II molecule. Picture modified from Jönsson et al. 1995.

At this point there was very little knowledge as to whether P70, Map or Eap were related. Palma et al. compared the NH2- terminal sequences of Eap and Map and found that the amino acid contents in these two proteins had a similarity of 97% implying a relationship between these proteins (82). This discrepancy was resolved when Hussain et al (2001) studied 140 clinical isolates of *S. aureus*. He found that Eap could be classified into three groups based upon PCR product sizes: group I (1.8kbp), group II (2-kbp) and group III (2.4kbp). Size difference was due to an insertional mutation of an additional base in the poly-A region, causing the premature termination. In the absence of this mutation all Eap and Map proteins have almost identical N-and C-terminal sequences. In addition, when *map* primers were used in a PCR analysis, 97.9% of *S. aureus* isolates are positive for Eap/Map but no Staphylococcus epidermidis isolates (49). Recently, Buckling et al. looked closer into this size difference and found that the *map*/*eap* genes are allelic variants of each other and that this gene is a phase variable gene that is able to express both long and short forms of the map/eap protein. If this is the case, it would be the first phase variation

mechanism described for *S. aureus* and also the first phase variation which results in length variation and not just in a switch on and off variation (13).

A great deal of interest has been focused on the biological functions of Eap. Palma et al (1999) had already shown that exogenously added Eap significantly enhanced the adherence of *S. aureus* to fibroblasts and epithelial cells, due to its dual affinity for both plasma proteins on the cell surface and the bacteria. These findings were confirmed through the construction of a stable Δ*eap* deletion mutant (Paper I).

If adherence of *S. aureus* to host components is the first step in infection, its ability to escape humoral immunity by internalization and intracellular survival might be the second most important function for long term persistence. Internalization of *S. aureus* into nonprofessional phagocytic cells is starting to be accepted as a fact (4, 56, 75, 118). FnBPA has been shown to be required for the internalization process into eukaryotic cells (23, 93, 106). Since Eap had an impact in the adherence process, the next step to be investigated was the potential role of Eap in the internalization of *S. aureus* into eukaryotic cells. *S. aureus* strain Newman AH12 (mutant for the *eap* gene) was used and found to have significantly reduced ability to internalize into fibroblasts and epithelial cells (Paper II).

The next natural step has been to investigate to which component (s) on the staphylococcal surface or structures on the eukaryotic cells Eap binds to. Several candidates have been suggested as binding structures for Eap on the bacteria. Flock et al. (2001) identified a 32-kDa neutral phosphatase located on the surface of *S. aureus* as a putative target for Eap (29). Kreikemeyer et al (2002) additionally identified at least two *S. aureus* cell surface proteins that appeared to be affinity targets for Eap (61). Furthermore Vuong et al. could show that Eap bind to teichoic acids on *S. aureus* (114). An interesting finding came in 2002 when Chavakis et al. found a potential receptor for Eap on the eukaryotic cells. They demonstrated an interaction between Eap and ICAM-1 in vitro, whereas no interaction could be seen with molecules such as Mac-1 or LFA-1 on leukocytes. In vivo, they could also show that Eap inhibited neutrophil recruitment during peritonitis, indicating that Eap could be a potential anti-inflammatory agent (15). The binding of Eap to ICAM-1 was shown to inhibit adherence of leukocytes to the endothelial tissue and thereby inhibits extravasation of leukocytes from the bloodstream into the site of infection (Paper III).

Lee et al. showed the same year (2002) that the presence of Map (i.e. Eap) interfered with T-cell proliferation, leading to chronic diseases such as arthritis, osteomyelitis and abscess formation (68). In addition, the direct influence of Eap on peripheral blood mononuclear cells (PBMCs) was studied. It was shown in a proliferation assay that at low concentrations Eap elicits a stimulatory effect on PBMCs and at high concentrations it had an inhibitory effect through induction of apoptosis of T and B cells (Paper IV).

The structural information for Eap was for a long time limited to the initial findings made by McGavin et al. He demonstrated that Eap consisted of 110- amino-acid domain repeated five to six times. Hussain et al. could explain the reason for the number variation in the repeats composition of the Eap protein (49,73). Recently Geisbrecht et al. (2005) determined the three-dimensional structure of three different Eap domains (repeats) and found an unexpected homology with the C-terminal domain structure of superantigens from *S. aureus* (34). Adding to these findings, Haas et al. found that Eap, CHIPS, staphylococcal superantigen like proteins 5 and 7 (SSL5 and 7), TSST-1 and SPE-C shared high degree of structure similarity with each other (38). It is interesting to point out here that despite their sequence homology to superantigens, proteins such as SSL proteins do not have superantigens properties. They also do not interact with T cells. On the contrary SSL5 and SSL7 have been demonstrated to interact with monocytes and dendritic cells (48). Recently SSL7 was found to bind to complement component C5 and IgA (63). The fact these proteins are present in all *S. aureus* strains indicate that they are important to the bacteria (48). The structural similarity found between Eap and these proteins may suggest that Eap either belongs to the superfamily of secreted bacterial toxins or superantigen-like proteins.

Genetic regulation

Regulation of most of the virulence factors of *S. aureus* involves two extensively studied regulatory systems, agr (accessory gene regulator) and sar (staphylococcal accessory regulator). The current view can be described briefly such that *sar* encodes a protein, sar A, which functions as a transcription factor, modulating virulence gene expression either directly or indirectly by affecting the agr system. Activated agr

activates production of an RNA molecule called RNAIII, this molecule is responsible for the up regulation of toxins, enzymes and extracellular proteins and represses genes encoding surface associated proteins. Agr expression is dependent on the growth phase of the bacteria. In the exponential phase, bacteria produces low levels of agr proteins which result in lower production of secreted proteins and high production of cell bounded proteins. When the concentrations of agr proteins increase (late exponential phase/ high density of bacteria), production of secreted proteins is increased and cell bound proteins decreased (14, 42). This may be of clinical importance for the bacteria since in an early stage of infection, i.e. bacterial growth phase, the expression of surface proteins facilitate binding to the host matrix proteins and favours successful colonization. Once the bacteria have become established and reached a higher density (late exponential phase), production of secreted proteins favours the spread to adjacent tissues. Harraghy et al. showed that in addition to agr and sar, production of Eap is also dependent on another regulatory system called sae (S. aureus exoprotein expression). Sae encodes a classical two-component system and is independent of the agr and sar system. Harraghy et al. could show that in a sae mutant, expression of Eap and Emp were severely repressed. On the other hand, sae was also reduced in the presence of glucose, suggesting that repression of Eap in glucose- containing medium may be the result of decreased expression of sae (39).

S. aureus immunomodulating proteins

A new family of proteins from *S. aureus* has been characterized under the denomination of microbial immunomodulatory molecule family, summarized in Table 1. Members of this family include superantigens (not included in this table), affecting T-cells and antigen presenting cells and, protein A having an impact on antibody response (95). The effect of SAK upon α-defensins was already mentioned early in this text (10, 99). Eap, Efb, CHIPS and SCIN are recent additions to this family. By virtue of the above already mentioned activities of Eap as an immunomodulating protein, this protein will likely be included in this family. Efb has also recently been found to interfere with the immune system. Efb binds the α-chain of C3 and thereby inhibits, both the classical and alternative pathway of complement activation. The blocking effect elicited by Efb was shown to inhibit complement mediated

opsonophagocytosis in a dose-dependent manner and that Efb could inhibit complement activity by blocking deposition of C3 or by preventing further complement activation beyond C3b (66, 67). Through this mechanism, Efb facilitates the establishment of the infections process by *S. aureus* and dilates the elimination of the bacteria.

Table 1. *S. aureus* Immunomodulating proteins

Protein	**Binding site**	**Reference**
Eap	Cell surface receptor ICAM-1	(15, 68), Paper III
Efb	Complement component C3	(66, 67)
CHIPS	Complement component C5a and formylated peptides	(21, 38, 96)
SCIN	Complement component C3 convertases (C4b2a and C3bBb)	(98)
SAK	Binding to α-Defensins	(10, 99)
Protein A	Fc portion of antibodies	(95)

CHIPS is an exoprotein, which has been shown to inhibit the response of neutrophils and monocytes to chemoattractants such as C5a and formylated peptides by binding to the corresponding receptors on the host cells (21,96). Both these chemoattractants have been shown to induce infiltration, trafficking and homing of leukocytes to the site of infection.

SCIN is the most recent addition to this family. It is another exoprotein that blocks human complement by specific interaction with C3 convertases, leading to substantial decrease in phagocytosis and killing of *S. aureus* by human neutrophils (98).

Preventing infections with *S. aureus*

Prevention of staphylococcal infections has been the aim of many studies involving *S. aureus*. Much has been done in this field, with variable results. The infectious process combines different steps starting with adherence, followed by penetration, multiplication, interference with the immune system and ending up in the worst case with tissue damage. With this scenario in mind, scientists have tried to block each of these steps and at the same time find a way to challenge our immune system to be better prepared for future infections with *S. aureus*.

Nasal carriage of *S. aureus* has been regarded as a source of increasing nosocomial infections. Recent studies have shown that wall teichoic acid (WTA), at the surface of the bacteria is essential for nasal colonization and mediates the interaction with the human nasal epithelial cells (116). Von Eiff et al. (112) showed in a prospective study that nasal strains and subsequent bacteremic strains had the same genotype in more than 80% of the cases. This indicated that patients colonized with *S. aureus* were at higher risk to be infected with their own nasal strain. Clinical studies using Mupirocin as a prophylactic agent to eliminate nasal carriage has resulted in a significant reduction of nosocomial *S. aureus* infection rate in different clinical populations (surgical, orthopedic, hemodialysis, etc) (9, 59, 94). It is important to point out that the prolonged use of Mupirocin has caused resistance to develop in the bacteria.

Many scientists have pursued the development of *S. aureus* vaccine, with small success. The repeated exposure to *S. aureus* as a member of the normal flora means that antibodies to *S. aureus* are prevalent in the human host. This explains the fact that infection with *S. aureus* does not protect against a new infection with *S. aureus*, making the development of vaccines a difficult task (65). Although a vaccine to prevent staphylococcal infections in the whole population has not been a main target, more vulnerable clinical populations could benefit from it. Different approaches have been taken to tackle this problem. Protein vaccines, composed of proteins that bind the extracellular matrix, for example fibronectin, fibrinogen and collagen have been investigated as candidates to protect against *S. aureus* infections (77, 78, 97); Toxoids and superantigens in mutant forms have been used in vaccines to generate protection (5, 79, 108) and RNAIII- activating Protein has been used as a vaccine in a mice model (2). All these attempts have resulted in limited protection against *S. aureus*

infections. A more promising study with regard to vaccination has been the conjugate vaccine, StaphVAX™. This consists of the two most prevalent capsular polysaccharides type 5 (CP5) and 8 (CP8), representing 85% of all clinical isolates, coupled to a carrier protein. This vaccine could elicit an efficient Th2 response and prevent *S. aureus* bacteremia for up to 10 months in hemodialysis patients (27).

The ability to control *S. aureus* will depend on many factors since this bacteria itself is very complex with numerous factors involved in its infectious process. A vaccine would need to contain a mixture of different components since many of the virulence factors have overlapping functions under the infectious process. A hypothetical choice could be a mixture of one or two adhesions (fibrinogen, fibronectin or collagen binding proteins), some mutated toxoids, and definitely CP5 and CP8. Other factors to be taken into account for the prevention of *S. aureus* infections will be the development of new antibiotic agents, implementation of more sophisticated prophylactic agents, better diagnostic tests to identify carriage and last, but not least important simple measurements such as hand washing that can have a huge impact on the cross- transmission of *S. aureus* in the hospital milieu (1).

Aims of this thesis

The overall aim of this thesis was to provide insight in the interaction between Eap from *Staphylococcus aureus* and the effects elicited by the protein on the human host.

The specific aims were:

1. To establish the role of Eap from *S. aureus* in the adherence and internalization process.

2. To determine the effect of Eap on adherence and migration of neutrophils on endothelial cells.

3. To determine the role of ICAM-1 in Eaps interaction with the endothelium.

4. To investigate the immune-modulation effects of Eap on peripheral blood mononuclear cells.

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Materials & Methods

The material and methods presented in this thesis are those that have been of most importance for each publication. All details can be found in the original papers.

Construction of Eap deficient mutant

To construct the *eap* deficient mutant an allele replacement method was used. The method is based on the insertion of a DNA fragment encoding a drug resistance into a gene of interest, in this case the *eap* gene. The goal was to disrupt the integrity of the *eap* gene with the DNA fragment. The inserted fragment blocks gene transcription and the drug resistance are used as a marker for the identification of the mutated cells.

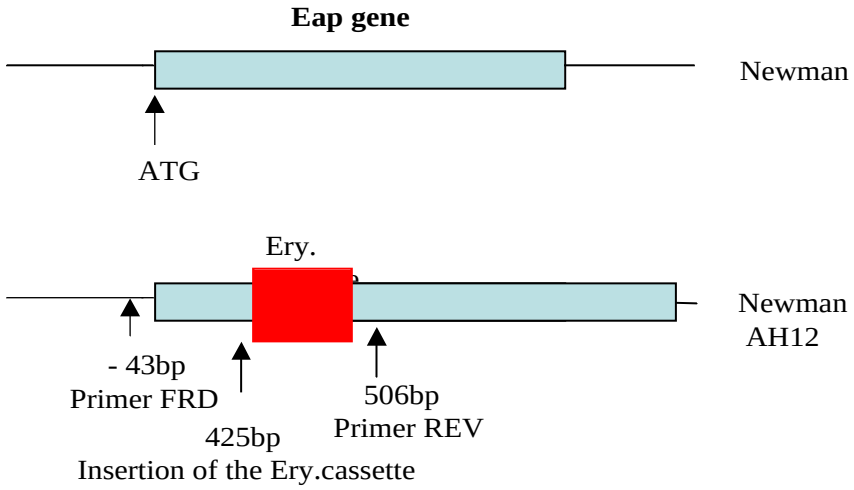

Figure 3. Genetic map of *eap* showing the place of insertion of the erytromicine cassette in strain Newman AH12, picture taken from Paper I.

The substitution of the wild-type allele with the mutated plasmid-located copy was achieved by a double recombination between homologous sequences; for example between the mutated (eap::Ery^R) and the wild type copy of the eap gene as shown in Figure 3. To confirm the allele replacement, genomic DNAs from *S. aureus* Newman and eap mutant AH12 were amplified; products of 2.1 kb and 3.4 kb, respectively were obtained as expected (**Paper I**).

Purification of Eap and pseudo Eap

Strain Newman and Newman AH12 were grown O.N at 37ºC in LB medium. The culture was centrifuged and FgBPs from the supernatant were isolated by affinity chromatography on Fg-Sepharose as described by Bodén and Flock (6, 7). Proteins were eluted with 0.7% acetic acid, dialyzed against 40mM phosphate buffer, pH 6.5 (buffer A) and subjected to FPLC on a Mono S column, using a gradient of 0 to 100% buffer B (1M NaCl in buffer A). Three peaks of proteins were eluted from strain Newman. The first one coagulase, the second one Efb and the third peak been Eap (**Paper I-IV**). The eluate (third peak) was dialyzed against PBS. The Eap-negative strain Newman AH12 was also used for purification of what we here call "pseudo Eap". The fraction corresponding to the third peak (pseudo Eap) from Newman AH12 was collected in the same way and was used as control (**Paper IV**).

Adherence and internalization of *S. aureus* strains into eukaryotic cells

Cells (Fibroblasts or epithelial cells) were seeded in 24-well culture plates and incubated at 37°C under 5% CO_2. For the binding assay, the following standard procedure was followed. Upon reaching confluency (2,5 x 10^5 to 3,0 x 10^5 cell/well), the cells were washed with the standard medium (Eagles medium without supplements), and 900 µl of the standard medium was added to the cells. Cells were inoculated with 100µl bacteria, 50 µl of strain Newman and 50 µl of mAH12 in a mixture, to obtain a final concentration of 10^7 bacteria per well to produce a multiplicity of infection (MOI) of 30 to 40. After incubation for 2 hours at 37°C, wells were washed 3 times with PBS. Trypsin was added to the wells to detach the cells, which were subsequently lysed by the addition of sterile water. The bacteria (both adherent and internalized) were serially diluted and plated on Blood agar plates. After 24 hours incubation, colonies were picked from the Blood agar plates onto LB plates containing erythromycin and incubated for 24 hours at 37°C to determine the ratio between the two strains (only mAH12 is erythromycin resistant). The exact ratio between the two strains in the mixture before adherence was determined in the same way, giving an initial ratio of 50:50 between the strains.

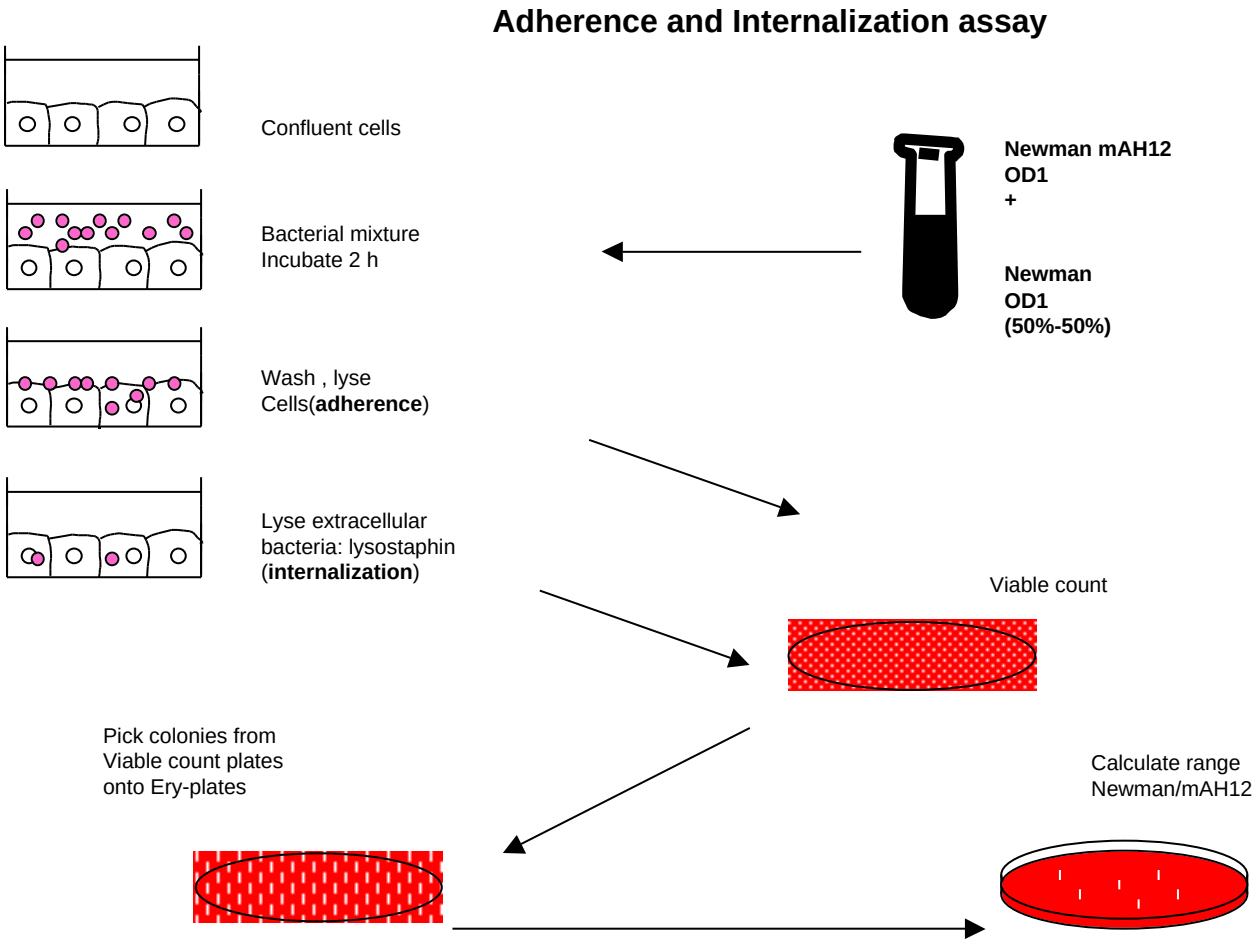

Figure 4. Adherence and Internalization of *S. aureus* strains Newman and Newman mAH12 into fibroblasts. Confluent layers of fibroblasts were inoculated with a bacterial mixture of strain Newman and Newman mAH12 in a 1:1 proportion. For internalization cells were further incubated with lysostaphin to kill the extracellular bacteria. Colonies were checked for erythromycin resistance by cultivation on erythromycin- containing plates to determine the proportion between the two strains.

For determination of internalization, lysostaphin at a final concentration of 20µg/ml was added for 30 min to kill extracellular bacteria before the trypsin step. Thus, only internalized bacteria are enumerated as shown in Figure 4.

Adherence and internalization assays were also performed in the presence of externally added Eap and antibodies against Eap (**Paper I and II**).

Static and flow adhesion assays

To find out the effects of Eap on the adhesion of neutrophils to non-stimulated or TNF-α-stimulated endothelial cells, static and flow adhesion assays were performed as described elsewhere (24). Human aortic endothelial cells (HAECs;) were cultured using EBM-2 medium supplemented according to the supplier (Clonetics). Human neutrophils were isolated as described previously (24), and used within 4 hours. To assess if Eap affects the static adhesion of neutrophils, a static adhesion assay was performed. Neutrophils were diluted to $5x10^5$ cells/ml in AIM-V, and 2 ml of cell suspension was added to confluent monolayers of HAECs and incubated for 5 minutes at 37°C. Prior to the assay, endothelial cells were incubated in medium alone (non-stimulated) or stimulated with recombinant TNF-α (20 ng/ml) for 6 hrs at 37°C. After 4 hours of incubation with TNF-α, Eap (30 μg/ml final concentration) was added and allowed to interact with the cells for an additional 2 hrs. Non-adherent neutrophils were carefully washed away and the cells in ten visual fields for each well were microscopically examined and counted.

In order to investigate whether Eap mediated all its blocking effect on neutrophil adhesion to endothelium via ICAM-1 binding (16), the blocking effect of Eap alone was compared to that of anti-ICAM-1 antibodies (8μg/ml final concentration) alone or in combination with Eap in static adhesion experiment. In order to investigate if Eap also affected the adherence of neutrophils under conditions of physiological flow, neutrophils ($5x10^5$ cells/ml) were perfused through a flow chamber over confluent monolayers of HAECs at 1 Dyne/cm^2 at 37°C. The endothelium was treated as described above, and cells in ten visual fields were counted for each well (**Paper III**).

Transendothelial migration

The effect of Eap on neutrophil transendothelial migration was investigated in a migration assay performed as previously described (40). HAECs were cultured on gelatin-coated filters for at least 5 days to reach confluence, and were stimulated as described above. Freshly isolated neutrophils were labeled with 10 μM Calcein AM in Krebs Ringer Glucose for 20 minutes at 37°C. Labeled neutrophils ($2x10^6$ cells) were

added to the upper compartment of the Transwell and allowed to migrate through the endothelium for 2 hrs at 37°C. As an attractant, 50 ng/ml of recombinant human IL-8 was added in culture medium to the lower compartment. Medium alone was added as a control for random migration. After migration, 0.1% Triton X-100 was added to each well to release Calcein from cells that had migrated. The concentration of Calcein in each well was measured by a Bio-Rad Fluor-S Max MultiImager. The value for maximal migration was obtained by measuring the fluorescence for cells directly added to the lower well. The percentage of migration was obtained by dividing migration with maximal migration (**Paper III**).

Peripheral blood mononuclear cell (PBMC) and Eap

PBMC were isolated from heparinized blood of healthy donors by Ficoll-Hypaque gradient centrifugation. The PBMC were cultured in RPMI 1640 medium. For stimulation of proliferation, PBMC were cultured with Eap at concentrations ranging from 0 to 81 µg/ml (final concentration). Sheep antibodies against Eap (0, 62 or 1000 µg/ml final concentration) or affinity purified human IgG against Eap (0 to 20 µg/ml final concentration) were used in combination with Eap (9 or 81µg/ml). PBMC ($2x10^6$ cells/ml) were cultured for 72h after which they were pulsed for 6h with [^{3} H] - thymidine. Phytohemmagglutinin-L (PHA) was used as a positive control for stimulation at a concentration of 2µg/ml. All samples were assayed in triplicates, and the data are presented as counts per minute (cpm). The experiments were repeated three times using cells from different individuals (**Paper IV**).

Apoptosis

PBMC were purified as described for the proliferation assay and challenged with different concentrations of Eap (0-81 µg/ml final concentration). After 72h of incubation, cells were analyzed using a FACS calibur flow cytometer (Becton Dickinson). Acquisition and processing of data from 15000 cells was analyzed using Quest software. Dot plots of forward scatter (cell size) vs. side scatter (internal complexity) were used to assess the viability of the cells. In order to determine the

effect of Eap on individual cells, 3- color flow cytometry was used. FITC-conjugated anti-human CD19 (B-cells) or CD3 (T-cell) were added to the cells, and incubated on ice for 30 min. For the analysis of apoptosis, cells were further washed once and stained with 5 µl of AnnexinV-biotin for 15 min at RT, followed by 1µl of streptavidin-APC incubation for 15 min. Finally, 1 µl of propidium iodide (PI) was added to the cells and analysis was carried out as described above (**Paper IV**).

Statistical methods

Unpaired student t-test and repeated measures one-way ANOVA was used to determine the statistical significance of the data.

Results

Role of Eap in adherence and internalization of *S. aureus* strain

A confluent layer of fibroblasts was inoculated with a mixture of *S. aureus* Newman / Newman AH12 (*eap* ::Ery^R) and incubated for 2 hours. Bound bacteria were detached and the ratio between the two strains was determined. After incubation with the fibroblast cells, a significant difference in adherence (P <0.01) of wildtype vs. mutant could be observed with the wild type adhering to a larger extent as shown in Figure 5.We also addressed the question whether not only adherence, but also internalization of the *eap*-mutant was reduced. Among the internalized bacteria in fibroblasts, the proportion of WT organisms was clearly dominating when compared to organisms of mutant strain AH12 as shown in Figure 5 (p<0.01). There was a 10- fold difference between strain Newman and AH12 in regard to internalization. Using the adherence and internalization assays on fibroblasts and epithelial cells, strain Newman was pre-treated with both externally added Eap and antibodies against Eap prior to addition to the cells. Both adherence and internalization of strain Newman were enhanced significantly by the presence of externally added Eap protein and reduced by the presence of the antibodies against Eap (**Paper I and II**).

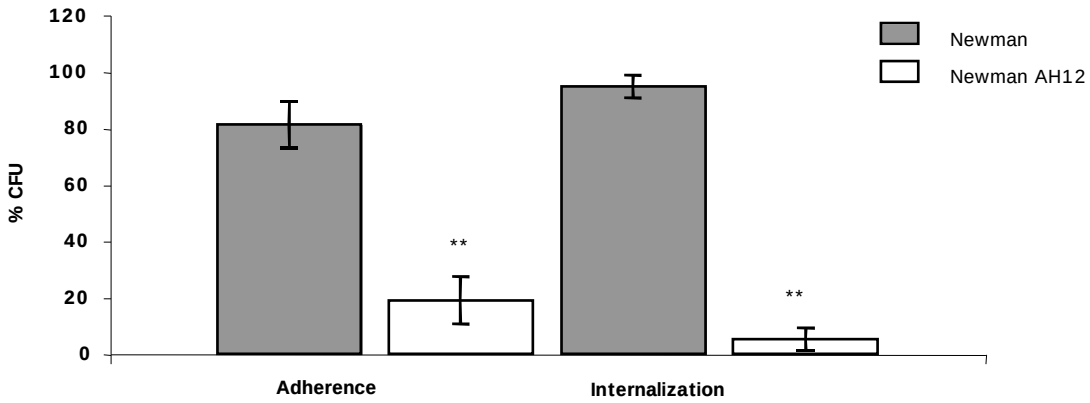

Figure 5. Adherence and Internalization of *S. aureus* strains Newman and Newman mAH12 into fibroblasts. Confluent layers of fibroblasts were inoculated with a bacterial mixture of strain Newman and Newman mAH12 in a 1:1 proportion and incubated at 37°C for 2 h. After adherence wells were washed, the bound bacteria were released by trypsination and plated on blood agar plates. For internalization cells were further incubated with lysostaphin to kill the extracellular bacteria. Colonies were checked for erythromycin resistance by cultivation on erythromycin-containing plates to determine the proportion between the two strains. The data are presented as the percentage of bound bacteria. The error bars show standard deviations; n= 3. **, P<0.01 (unpaired *t* test).

Effect of Eap on neutrophils

We also looked at the effects of Eap on the binding of neutrophils to endothelial cells by simulating an inflammatory process (TNF-α stimulated HAECs). To investigate these effects we used a static, flow and transendothelial migration assay. We found that regardless of the condition of the HAECs, Eap significantly ($p < 0.05$) inhibited binding of neutrophils to endothelial cells under static conditions. As expected, neutrophil adhesion was lower to non-activated HAEC than to HAECs activated with TNF-α. The reduction of neutrophil binding exerted by Eap was more pronounced on activated than on non-activated endothelial cells as shown in Figure 6 (**Paper III**).

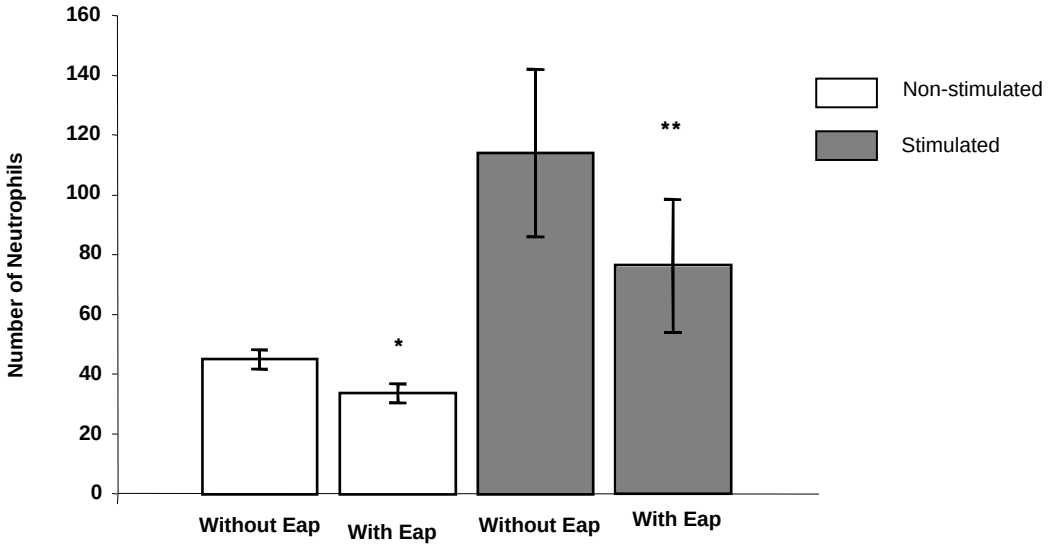

Figure 6. Static adhesion assay. Neutrophils ($1x10^6$ cells) were added to confluent monolayers of endothelium and allowed to adhere for 5 min at 37°C. Endothelial cells were treated at 37 °C with medium alone (white bars) or TNF-α for 6 hours (shaded bars) prior to the assay. After 4 hours, Eap (30 μg/ml final concentration) was added to some wells and further incubated for 2 hours. The data are presented as mean ± SEM of five experiments. Statistical difference was determined by student's t-test, * $p<0.05$ and ** $p<0.01$.

Since ICAM-1 had been proposed as a putative receptor for Eap, we used anti-human ICAM-1 antibodies and assessed the degree of blocking to that of Eap. Only TNF-α-stimulated endothelium was used in this experiment and after 4 hours of TNF-α treatment, anti-human ICAM- 1 antibodies, were added. The blocking effect of ICAM-1 antibodies and Eap were of similar magnitude and no additive or synergistic effects on blocking could be detected when antibodies and Eap were used together

($p< 0.05$), as shown in Figure 7. This strongly implies that there is a common target for the two blocking agents, ICAM-1.

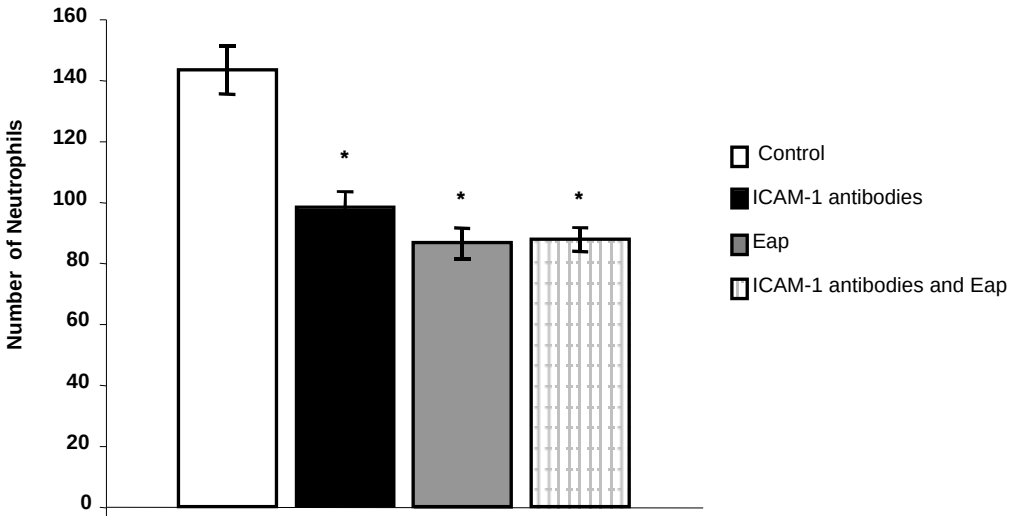

Figure 7. Static adhesion assay in the presence of antibody. In the antibody blocking assay all endothelial cells were treated at 37°C with TNF-α for 6 hours prior to the assay. After 4 hours some wells were pre-incubated with ICAM-1 antibodies for 20 min. Eap (30 μg/ml final concentration) was added to some wells and further incubated for 2 hours. Cells in ten visual fields were counted for each well. The data are presented as mean ± SEM of four experiments. Statistical difference was determined by student's t-test, * $p<0.05$.

Also in the flow adhesion assay, Eap significantly ($p< 0.01$) inhibited the neutrophil adhesion, both to non-stimulated and TNF-α stimulated endothelium (**Paper III**).

In the migration assay, we observed that Eap inhibited the migration of neutrophils across stimulated endothelium in the presence or absence of human IL-8 ($p<0.05$). However, no effect of Eap on the migration across non-stimulated endothelial cells was detected. Eap thus block only the transendothelial migration, which is dependent on stimulation and expression of ICAM-1 (40) as can be seen in Figure 8, (**Paper III**).

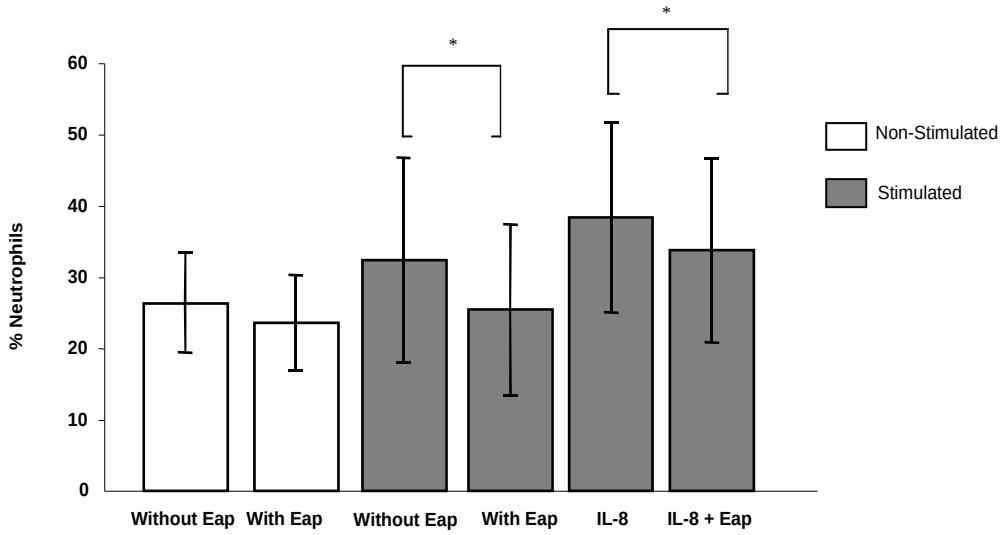

Figure 8. Migration assay. Human neutrophils ($2x10^6$ cells) were allowed to migrate across monolayers of HAECs for 2 hours at 37°C**.** Endothelial cells were treated at 37°C with medium alone (white bars) or TNF-α for 6 hours (shaded bars) prior to the assay. After 4 hours of incubation, Eap (30 μg/ml final concentration) was added to some wells and further incubated for 2 hours. As an attractant recombinant hIL-8 in culture medium was added to the lower compartment. The value for maximal migration was obtained by measuring the fluorescence of cells directly added to the lower compartment of the Transwell® plate. The percentage migration was obtained by dividing the observed migration with maximal migration. The data are presented as mean ± SEM of five experiments. Statistical difference was determined by student's t-test, * $p<0.05$.

Dose- dependent differential effect of Eap on PBMC.

To assess the effect of Eap on human immune cells, PBMC were stimulated with a *S. aureus* supernatant containing superantigen in the presence or absence of Eap (30 μg/ml). Eap was found to significantly inhibit the proliferative response induced by supernatant ($p<0.01$) (**Paper IV**). For this reason PBMC were cultured in the presence of different concentrations of Eap (0-81μg/ml). Eap was found to have a stimulatory effect at concentrations ranging from 3 μg/ml to 9 μg/ml ($p<0.05$),as shown in Figure 9, while at higher concentrations an inhibitory effect was seen ($p<0.01$) when incubated for 72 hours. These dose-dependent stimulatory and inhibitory effects were seen regardless of cell proliferation time, 48, 72 or 96 hours (**Paper IV**).

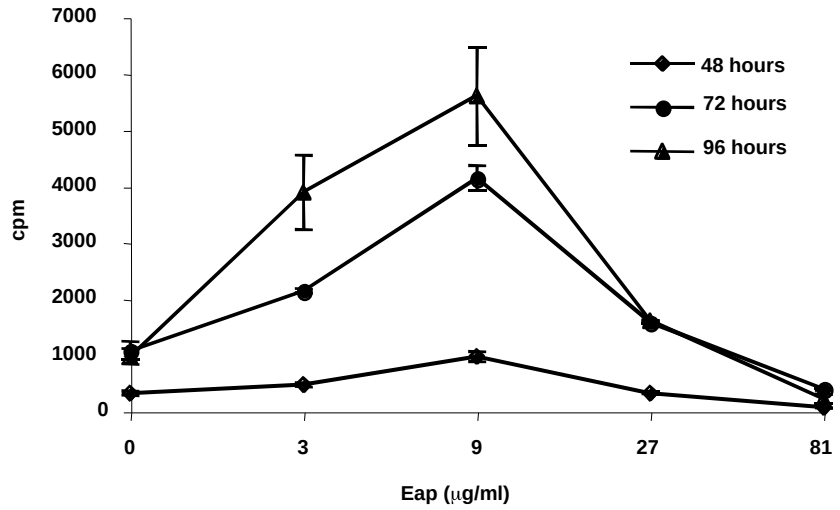

Figure 9. Dose dependent effect of Eap on PBMC. PBMC were stimulated with indicated concentrations of Eap and at three different incubation times with Eap (48h, 72h, and 96h). Data are presented as the mean number of cpm values from five experiments for 72 hours and one representative experiment of two for 48 and 96 hours. Error bars indicate SEM. Statistical difference was performed on data from the 72 hours time point. Values for Eap at 9 μg/ml was significantly higher than values for Eap at 0μg/ml ($p<0.05$) or at 81μg/ml($p<0.01$), determined by repeated measures one-way ANOVA.

Since antibodies against Eap (sheep antibodies) had shown an inhibitory effect in the adherence and internalisation assay we looked into the effect of these antibodies in our proliferation assay. PBMC were incubated with either the stimulatory (9μg/ml) or the inhibitory (81μg/ml) concentration of Eap together with increasing concentrations of sheep antibodies against Eap. Pre-immune sheep IgG was used as negative control, and no blocking effect upon Eap was detected. The stimulatory effect elicited by Eap at 9μg/ml was significantly blocked ($p<0.05$) by antibodies at 62μg/ml. The inhibitory effect of the higher Eap concentration (81μg/ml) turned into a stimulatory effect by antibodies, presumably due to reduction of the Eap concentration, into the stimulatory concentration range (**Paper IV**). A preparation of human IgG was found to contain antibodies against Eap (data not shown) although at a low level. Such antibodies were purified by affinity chromatography, and found to inhibit the stimulatory effect of Eap on PBMC at concentration of 5μg/ml ($p<0.05$) and 20 μg/ml ($p<0.01$) as shown in Figure 10 (**Paper IV**).

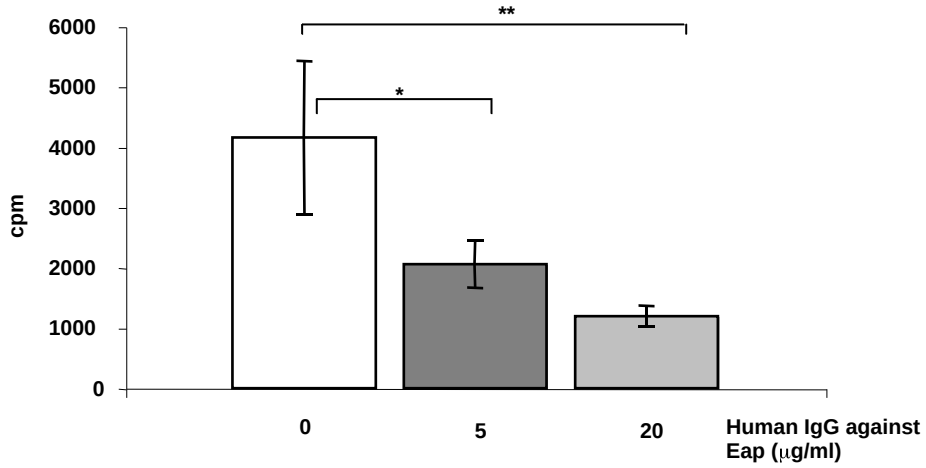

Figure 10. Effect of anti-Eap antibodies. PBMC were incubated for 72h with Eap at 9μg/ml with human affinity purified IgG against Eap. Data is presented as the mean number of cpm values of three experiments performed in a and the mean number of cpm values from three experiments performed with human IgG against Eap in b. Error bars indicate SEM. Statistical difference was determined by Students t test *, $P < 0.05$; **, $P < 0.01$.

Eap induces apoptosis in human B- and T-cells

PBMC cultured together with Eap were characterized for apoptosis merely by FACS analysis. In the presence of increasing concentrations of Eap, the proportion of the apoptotic/necrotic cell population dose- dependently increased. Cells were stained with Annexin-V and propidium iodide (PI). Redistribution of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane occurs early and is a hallmark of an apoptotic process. Annexin-V has a high affinity for phosphatidylserine and was used to distinguish early apoptotic cells from late apoptotic cells. We could show a clear shift from viable cells, 0μg/ml of Eap to nonviable cells (apoptotic/necrotic) with increasing concentrations of Eap 9μg/ml to 81μg/ml (**Paper IV**).

To investigate the effect of Eap on specific cell types, cells were differentially stained for markers of B- or T-cells. A clear shift from viable cells to apoptotic/necrotic cells was seen in both cell types as shown in Figure 11. An increase from 28% Annexin-V/PI positive B-cells to 76% when Eap concentrations were increased from 0μg/ml to

81µg/ml was demonstrated (Figure 11A). Similarly, an increase in apoptotic T-cells from 6% to 42% using the same Eap concentrations was found (Figure 11B).

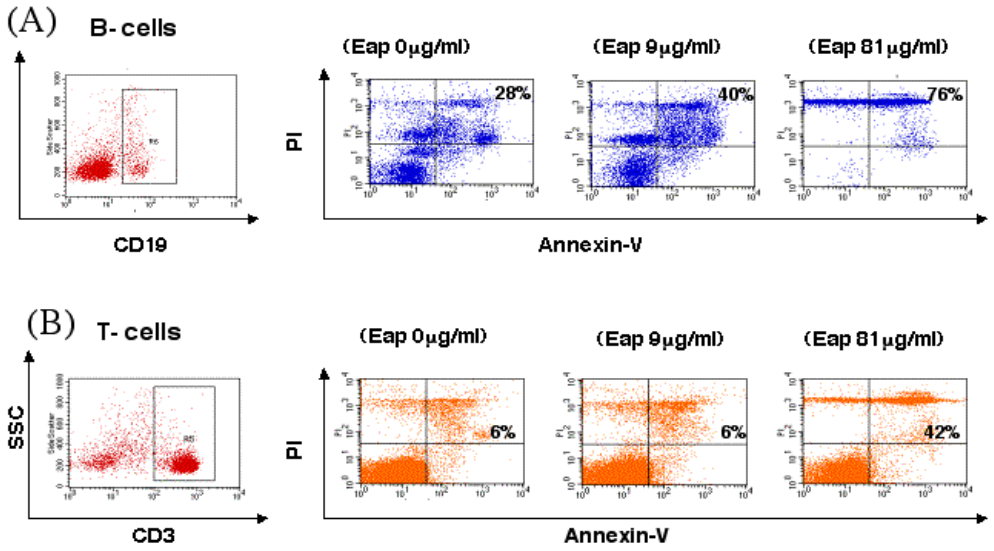

Figure 11. Eap induced apoptosis on B-and T-cells. PBMC were incubated for 72h with rising Eap concentrations prior to staining with Annexin-V, PI and FITC- conjugated anti-human CD19 (B-cells) or CD3 (T-cell). The total population of either T- cells or B-cells was gated and from these respective populations dot plots of Annexin-V versus PI were generated, in order to determine the percentage apoptotic cells present. The total population of either B-cells or T-cells in the absence of Eap is shown in Fig.11A and 11B respectively. The percentage of T-cells or B-cells that are double positive for Annexin-V/PI in the absence of Eap, Eap at 9µg/ml and Eap at 81µg/ml are shown. Dot plots shown are representative of three experiments performed.

Discussion

In this thesis we have investigated the interaction between Eap and the human host. Our investigation started with the findings made by Palma et al. (82) in which Eap was characterized as a broad spectrum binding protein for host molecules and that externally added Eap enhanced the adherence of *S. aureus* to eukaryotic cells. In order to broaden the knowledge about Eap we constructed a genetically defined *S. aureus* mutant with a functional deletion of *eap* (**Paper I and II**). The successful construction of this stable Δ*eap* deletion mutant, AH12 (eap::Ery^R), in strain Newman, allowed us to study in a more precise manner the role of Eap. Both strains (wild type Newman and AH12) bound equally well to fibronectin and fibrinogen coated surfaces, both strains could rebind Eap to their surface, however in contrast, strain AH12 adhered significantly less to cultured fibroblasts. This indicated that Eap was important for the binding of the bacteria to eukaryotic cells but that the binding of Eap to bacteria itself or to matrix binding proteins was independent of endogenously produced Eap (**Paper I**). Using mutant strains deficient in *fnbA* and *fnbB* (35) or mutants deficient in *clfA* and *clfB* (25), it had already been shown that these genes gave rise to the major structures for functional binding of their respective matrix protein. Since strain AH12 expressed functional ClfA and ClfB, as well as FnbA and FnbB, it was not surprising to observe that the Eap-mutant interacted equally well with immobilized Fg and Fn as the wild type strain.

It is important to indicate that although adherence was not abolished in the absence of Eap it was significantly compromised (**Paper I**). Adherence is the first step in the infectious process and is without doubt the result of additive effects of a number of different ECMBPs, including Eap. Dziewanowska et al. could show that a deletion of the fibronectin-binding protein produced only a 40% reduction with regard to adherence (23). Many of the ECMBPs have overlapping functions, which are difficult to study by the loss of a single protein, as is the case with strain Newman AH12. Studies of double or triple mutants may help to elucidate the real impact of Eap in the binding of *S. aureus* to eukaryotic cells.

Having established the effect of Eap in the adherence of the bacteria to eukaryotic cells, the next step was to determine the impact in the more complicated process of

internalization. We could show that strain Newman could internalize into eukaryotic cells to a greater extent than strain Newman AH12 and that externally added Eap enhanced the internalization into eukaryotic cells of strain Newman, strain Newman mAH12, clinical isolates and even the distant related strain *S. carnosus* TM300 (**Paper II**). By this time it had already been shown that FnBPs played an essential role in the internalization process by promoting an actin-mediated phagocytosis of the adherent bacteria. In fact, by using FnbA and FnbB deletion mutants, complemented strains, D1-4 repeat peptide, and heterologous complementation in *S. carnosus*, it was demonstrated that FnBPA and FnBPB are, to different extents, both necessary as well as sufficient for a complete internalization process (23,106,107). However these experiments were performed in an Eap-proficient background, which therefore does not rule out the possibility that Eap has an enhancing effect on the internalization process.

Although FnBPs obviously play a crucial role in the internalization process, bacteria lacking FnBPs could still be internalized at a lower rate. Furthermore, no correlation was found between adherence ability and the amount of FnBPs produced by some *S. aureus* strains (43), and Fn binding capacity only partly correlated with the ability of various strains of *S. aureus* to be internalized (23, 93). Dziewanowska et al. also noted that there is not a complete correlation between the efficiency in adherence of *S. aureus* to immobilized fibronectin and the level of internalization into Mac- Tcells. This suggests that factors other than fibronectin binding could affect internalization in some isolates of *S. aureus* (23). In addition to *S. aureus,* several other Gram -positive bacteria, including *Listeria monocytogenes*, *Streptococcus pyogenes* and *Enterococcus faecalis*, evade host immunity by internalization (52, 53, 101, 117). *Listeria monocytogenes* uses two invasion proteins for entry into mammalian cells, internalin A (InlA) and internalin B (InlB). InlA is a transmembrane cell adhesion protein (74) promoting entry into the enterocyte-like epithelial cell line Caco-2 (31). InlB interacts with the mammalian protein gC1q-R,(12) and is needed for entry into cultured hepatocytes and epithelial or fibroblast-like cell lines (22, 32, 36, 51, 69). Interestingly, InlB is not only cell associated but also found in culture supernatants of *Listeria monocytogenes (69)*, analogous to Eap. It was also seen that InlB when added to the bacteria could rebind and enhance the internalization of *Listeria monocytogenes* into mammalian cells (11, 12). Thus, the internalization process of *Listeria*

monocytogenes is a multifactorial event, as we believe the *S. aureus* internalization process to be. These findings indicate that the internalization process for *S. aureus* is complex and probably involves more than one factor. Therefore, in analogy to Eap, bacterial internalization mechanisms in general may critically depend on the presence of secreted molecules in addition to proteins covalently bound to the cell wall.

At the time, the finding that Eap was involved in internalization process appeared a bit controversial. Until then it was accepted that the only proteins involved in promoting internalization were FnBPs. The situation was clarified by the findings of Grundmeier et al. who showed that while strain Newman produced FnBPs these proteins were defective as a result of point mutations, leading to the loss of all FnBPs- dependent functions (37). In the absence of functional FnBPs, as in the case of strain Newman, Eap, which is highly expressed in this strain (49), appears to compensate for adherence to fibroblasts and partially compensate for the loss of fibronectin binding and mediate invasion. It is important to point out that Eap is found it almost 97% of all *S. aureus* isolates (49), and it is most unlikely that only strain Newman would use Eap for the internalization into eukaryotic cells. Furthermore, experiments with *S. carnosus* showed that internalization can occur without Eap or without FnBP, but either of these proteins must be present to allow internalization (**Paper II**). These data provide evidence that Eap complements the internalization pathway of FnBPs rather than being separate events along the same pathway. Since internalization provides the bacteria with many benefits it is very wise from an evolutionary point of view to have more than one pathway for this process, which permits *S. aureus* to protect itself more efficiently from host defense and antibiotic treatment. Future studies are required to evaluate in more detail the relative contribution of difference of FnBPs and Eap in the internalization process.

The second part of this thesis has highlighted the direct effect of Eap on the immune system. Here we focused on and further investigated the interaction between Eap and the cell adhesion molecule ICAM-1. We demonstrated that Eap inhibits the binding of neutrophils to the endothelium under static and dynamic flow conditions and also inhibits transendothelial migration in an in vitro model. Human antibodies against ICAM-1 offered no additional blocking effect over Eap, confirming that Eap´s blocking effect was solely ICAM-1 dependent (**Paper III**). Neutrophil adhesion to the

endothelial cell lining the blood vessel wall and the subsequent migration of the neutrophils into the underlying tissue are important elements of innate and adaptive immunity. The strong adhesion of the neutrophils to the endothelial surface is a prerequisite for migration across the endothelium and is mediated via endothelial ICAM-1 and VCAM-1 (vascular cell adhesion molecule). Whereas VCAM-1 has only been demonstrated to play a role in the migration of monocytes across the endothelium, there is general agreement that the β2 integrins LFA-1 and MAC-1 and their corresponding ligand ICAM-1 on the endothelial cells are essential for migration of all neutrophils across the endothelium (16, 26).

The immuno-modulating properties of Eap are not unique for this *S. aureus*. In the introduction I have described proteins such as Chips Efb and SCIN from *S. aureus* exerting effects on the immune response. Furthermore, several other bacterial species also influence the immune system, either by increasing transendothelial migration through an up-regulation of adhesion molecules such as VCAM-1 and ICAM-1 on endothelial cells (*Salmonella typhimurium, Streptococcus mutans* and *Borrelia burgdorferi*) (33, 102, 111) or by blocking migration through binding to Mac-1 on leukocytes exemplified by the adhesin Filamentous hemagglutinin (FHA) from *Bordetella pertussis* (100). Targeting of the immune system seems to be a common defense mechanism used by bacteria in order to gain advantage in the infectious process and *S. aureus* seems to be no exception. The inhibitory effect elicited by Eap on the migration of neutrophils across endothelial cells may give *S. aureus* anti-inflammatory properties.

Having established the inhibitory impact of Eap on transendothelial migration most likely through the binding to ICAM-1 we looked closer into other processes where ICAM-1 had key functions and therefore could be affected by the presence of Eap and offer an advantage to the bacteria. In a proliferation assay we showed that Eap had a concentration-dependent effect on PBMCs healthy donors. At low concentrations Eap elicited a stimulatory effect on PBMCs and at high concentrations it had an inhibitory effect through induction of apoptosis of T and B cells. Antibodies against Eap could block the effects elicited by Eap upon PBMCs (**Paper IV**).

Two aspects of Eaps biology can be deduced based on these findings. Firstly, the concentration–dependent effect, are not unusual for *S. aureus* toxins. The α-toxin

from *S. aureus* has been shown to induce apoptosis or necrosis on endothelial cells, depending on the extracellular concentrations of the toxin. Lower concentrations induce apoptosis whereas higher concentrations induce necrosis of endothelial cells (57, 76). Similarly, TSST-1 has also been shown to have different effects on host cells, dependent on concentration. At low concentrations, TSST-1 stimulates Ig synthesis by PBMC from normal subjects and at high concentrations TSST-1 induces B-cell apoptosis (46). Interestingly, several of the concentration-dependent effects are linked to apoptotic/necrotic consequences, which is likely to be an important event in pathogenesis, considering that *S. aureus* has evolved several ways to induce apoptosis/necrosis. The question that can be asked is Eap a toxin or those Eap belong to immune evasive molecules such as CHIPS or SSL proteins? Although the crystal structure and sequence of Eap reveals it a considerable homology to the structure of superantigens from *S. aureus*, Eap also reveals homology to these other proteins (CHIPS, etc). Until know there is not enough data to claim that Eap is a superantigen and further studies are needed in this field (34,38). One should keep in mind that only nanogram quantities of a conventional superantigen are required, whereas with Eap, micrograms are needed in order to achieve a response. The relative expression of superantigens and Eap in patients is currently unknown. However it seems likely that there are substantial differences in superantigens concentrations as compare to Eap. I am more inclined to propose that the concentration dependent effect we see is due to bacterial concentration. A likely hypothetical model would be that at low concentrations, during the early stage of infection characterized by a lower bacterial density, Eap is present at levels corresponding to the stimulatory concentration. This would, together with the action of other bacterial factors, recognized by the immune system, result in an expansion/proliferation of human immune cells. In contrast, at a later stage of infection when the bacterial population is dense, Eap, along with other secreted staphylococcal products, may accumulate to reach levels in the extra cellular environment that are capable of triggering apoptosis and necrosis.

The second aspect that can be deduced is the direct binding of Eap to PBMCs. The receptor involved in this event is, as of yet unknown. One candidate is ICAM-1. In addition to trigger neutrophil migration, ICAM-1 is an essential molecule involved in the process of antigen presentation (19,47). Binding of ICAM-1 on antigen presenting cells (APCs) and LFA-1 expressed on T-cells leads to the induction of cell surface

and intracellular molecular events that facilitate antigen presentation to T cells. Briefly, one can divide the process of antigen presentation into two signals, one received by a T-cells via T-cell receptor (TCR) binding to MHC and a second signal received via accessory or co-stimulatory molecules, LFA-1 versus ICAM-1 (19, 64). Furthermore, it has been shown that the lack of ICAM-1 on APCs leads to poor T-cell activation and proliferation in vitro and in vivo. In addition to this it has been shown recently that ICAM-1 has a decisive effect during the course of T cell priming. Signals provided by ICAM-1 on APCs can have long-term consequence on cell fate determination, in particular in the composition of T memory cells (86).

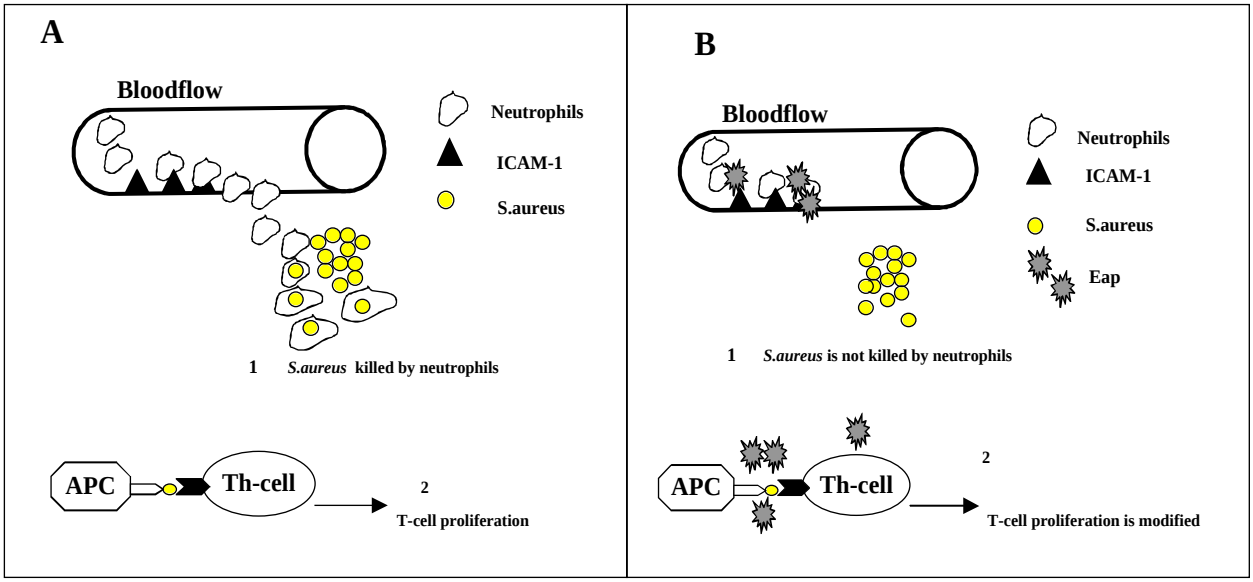

Figure 12. Schematic picture of the proposed effects of Eap on transendothelial migration and T-cell proliferation. These two events are not necessarily happening simultaneously but for explanatory reason are shown together.
Under normal conditions, neutrophils move rapidly through the blood vessels. During an infection, macrophages and other cells, release a variety of cytokines TNF-α , IL-1, gamma interferon (IFN-γ) and IL-8, which, stimulate neutrophils to leave the bloodstream and migrate to the site of infection. The release of these cytokines stimulates endothelial cells to produce a set of surface proteins such as selectins (slows the neutrophils down) and ICAM-1 (generate a strong attachment between neutrophils and the endothelium). ICAM-1 stops the movement of the neutrophils and causes them to flatten against the blood vessels, which is a prerequisite for neutrophil migration from the bloodstream into the infected tissue. Once neurophils reach the infected tissue they start killing bacteria (**A1**). In the tissue APCs (ex. macrophages) interact with Th-helper cells, resulting in T-cells proliferation (**A2**).
In the presence of Eap, neutrophil migrations to the infected tissue is impaired, since Eap binds to ICAM-1 and inhibits the strong bind of neutrophils to the endothelium (**B1**). Eap, impaired T-cell proliferation, by a mechanism yet not known (**B2**). ICAM-1 is an important molecule involved in the process of antigen presentation, which leads to T-cell proliferation and Eap may interfere in this process (19, 47).

Verdrengh *et* al. demonstrated the impact of ICAM-1 on infection in an in vivo model. When infected with *S. aureus*, knock-out ICAM-$1^{-/-}$ mice developed less frequent and less severe arthritis than their wild-type littermates (110). Furthermore, Lee *et* al. showed that Map^{-} bacterial strains (i.e.) less frequently cause arthritis and osteomyelitis (68). These data indicate that the absence of ICAM-1 or the presence of Eap leads to a similar outcome during the infectious process of *S. aureus* and therefore ICAM-1 may be a putative receptor involved in the responses to Eap. The presence of Eap during *S. aureus* infection and its effect on transendothelial migration and antigen presentation to T cells will weaken the protective cellular immunity, which is the main mechanism used by the host to clear bacterial infections as exemplified schematically in Figure 12.

Evidence that Eap is expressed in vivo and can challenge the host immune cells was obtained by determining the presence of anti-Eap antibodies in sera. Patients with ongoing staphylococcal infections had significantly increased acute-phase antibody levels against Eap when compared to the healthy controls, (Haggar et.al, unpublished data). The levels of antibodies against Eap increased even more during the convalescent phase, 14 to 30 days after onset of disease. In all experiments where antibodies against Eap were used, they could block the effects of Eap. Human IgG against Eap could block the stimulatory effect on PBMC (**Paper IV**), and sheep IgG against Eap blocked adherence and internalization of *S. aureus* into eukaryotic cells (**Paper I and II**). The human anti-Eap antibodies (**Paper IV**) were purified from Intravenous Ig preparations (IVIG), which are prepared from large pools of human plasma, containing considerable amounts of neutralizing anti–superantigen antibodies. Treatment with IVIG has been shown to reduce cytokine secretion, bacterial load and mortality in streptococcal and staphylococcal superantigen mediated toxic shock syndrome. The clinical efficacy on staphylococcal toxic shock has so far only been based on a few case reports (3, 20, 48, 60, 80). Since antibodies against Eap have had positive result in blocking the effects of Eap, one could speculate that these antibodies could be used in a therapeutically context to limit *S. aureus* infectious. A hypothetical treatment could be antibodies against Eap in combination with antibodies against other ECMBPs, which could be a putative treatment or prophylaxis in patients undergoing operations were the risk of getting infected with *S. aureus* is high. On the

other hand, since Eap has been shown to have anti-inflammatory effects, it might be used as a treatment against chronic inflammatory diseases.

Here we have presented the functional characteristics of Eap and looking at all the various functions of Eap, an obvious question remains, whether or not Eap is a multifunctional protein or are all the functions we have studied the result of a single event (binding to a particular receptor) with multiple consequences. Further studies are required to clarify this important issue.

Taken together, our findings in **Paper I** to **IV** in this thesis we conclude that Eap from *S. aureus* has several biological functions of importance in pathogenesis of *S. aureus*, (i) adherence and (ii) internalization into host cells, as well as (iii) immunomodulatory effects. Altogether these effects are likely to have a profound impact on the innate and adaptive immune response of the host during an *S. aureus* infection.

General Conclusions

In this thesis we have characterized **Eap**, an extracellular protein from *Staphylococcus aureus*. The ability to establish a niche in the host is a crucial step in the bacterial pathogenesis. The second most important step in the infectious process is the capacity of the bacteria to reproduce, disseminate and evade the host immunity. *S. aureus* intracellular location has been associated with all these aspects adding to these relapsing infections, and avoiding antibiotic treatment. We have shown that although adherence to eukaryotic cells is a multifactorial process, Eap has a significant role in this process. Having established the role of Eap in adherence we also showed that Eap plays a significant role in the internalization of the bacteria into eukaryotic cells. In addition the presence of antibodies against Eap blocked both the adherence and internalization process. From our studies we can conclude that Eap from *S. aureus* has an additive role in the adherence process and a complementary role in the internalization process in parallel with the internalization pathway shown for FnBPs.

In the second part of our studies, we have confirmed the findings that Eap has immunomodualting properties. The extravasion of neutrophils into the nearby tissue and the proper stimulation of T-cells are of pivotal importance for the host during an infectious process. The presence of Eap impaired both of these functions, in fact, the presence of Eap led to inhibition of transendothelial migration of neutrophils and additional, Eap showed a concentration dependent effect (stimulatory/inhibitory) on PBMCs, which culminated in the induction of apoptosis of T and B cells. We believe that the outcome of both these results are probably due to the binding of Eap to ICAM-1. Eap, through is binding to ICAM-1, is able to have an impact on key functions of both the innate and adaptive immune response.

In conclusion, Eap seems to be an important immunomodulating protein, which ought to be taken into account for future treatment strategies for *S. aureus* infections.

Future Prospects

It is well establish that Eap is composed of 110- amino-acid domain repeated five to six times (73). The three-dimensional structure of three different Eap repeats have been determined and found to have structural homology with superantigens, in addition Eap share a sequence homology with immune evasive molecules such as CHIPS and SSL proteins 5 and 7 (34).

Our interest has been to look closer into the biological properties of the single repeats of Eap. In order to do so, we have investigated the functions, either of individual repeats alone or in combinations of two or three together (unpublished data). Until now we have demonstrated that none of the repeats by themselves had a biological function in the aggregation or the adherence and internalisation process of *S. aureus* (unpublished data). One can speculate that for aggregation, adherence and internalisation more than one binding site is required and a single repeat is unable to achieve the biological function of the whole protein with multiple binding sites. On the other hand, proliferation of PBMCs in the presence of Eap or Eap-repeats, produced the same profile. The outcome of this result is not yet fully understood. The characterization of the single repeats of Eap is an ongoing project in our lab.

In addition to these findings many other questions remain to be clarified and going to be issues for future studies. How does Eap elicit apoptosis, which pathway is used? Does Eap elicit a cytotoxic or cytolytic effect and is there a natural receptor for the Eap protein on T or B-cells and how does Eap affect the infectious process in vivo? Is Eap mainly an anti-inflammatory protein or are we dealing with another kind of superantigen from *S. aureus*? Is Eap an SSL-protein? These and many other aspects ought to be considered. Eap from *S. aureus* is without doubt a protein with a great potential for future research in the area of *Staphylococcus aureus* infection.

Populärvetenskaplig sammanfattning

Många bakterier lever i harmoni med oss, det finns en balans mellan bakterierna och vårt immunsystem. Immunsystemet har uppstått för att skydda oss mot angrepp från yttre faktorer, t.ex. bakterier, virus och svampar. När en person på grund av andra sjukdomar har nedsatt immunförsvar rubbas balansen mellan människan och bakterierna, bakterierna tar över och infektionen är ett faktum. Detta gäller för de flesta bakterier och stafylokocker är inget undantag. *Staphylococcus aureus* är den vanligaste orsaken till infektioner man drabbas av under en sjukhusvistels. De kan orsaka livshotande infektioner, vilka kan vara mycket svårbehandlade då stafylokocker lätt utvecklar resistens mot flertalet antibiotika. Bakterierna klarar i allt större omfattning att överleva den antibiotikabehandling som ska skydda människan mot infektioner. Eftersom bakterier utvecklas och framförallt anpassar sig relativt snabbt till de nya antibiotika som finns tillgängliga pågår en ständig kapprustning mellan människan och bakterierna. Det är ett av skälen till att man har börjat titta närmare på andra sätt att förhindra stafylokockinfektioner utöver antibiotikabehandling.

Vilken exakt mekanism *S. aureus* använder sig av för att fästa till värden är inte helt kartlagt. Bakterien måste dock först kunna fästa sig till värdens vävnad för att sedan kunna kolonisera. För att kunna fästa sig till värden och fullborda detta avgörande steg har *S. aureus* ett antal matrixbindande proteiner (ECMBP= extracellular matrix binding proteins). Dessa proteiner gör att bakterier kan fästa sig till värdceller och i vissa fall kan detta leda till internalisering in i cellerna som i sin tur medför att bakterier ungår att bli upptäckta.

Eap, är huvudsakligen ett extracelluärt protein, som utsöndras från bakterie, (70%) och till en mindre grad hittar man också Eap bunden till bakterieytan (30%). Eap kan bilda oligomerer (proteinklumpar) med sig själva och kan agglutinera bakterien (bilda bakterieklumpar). Eap har också förmågan att binda tillbaka till bakteriens cellyta. Eap kan binda till sju plasmaproteiner, bland annat fibrinogen, fibronektin och protrombin.

I denna avhandling har jag kunnat visa att Eap ökar bindning och internalisering av *S. aureus* till humana celler (**Publikation I och II**). Detta kan bero på att vävnaden eller cellerna är täckta med plasma proteiner som Eap kan binda till. Genom att Eap även binder till bakterieytan, fungerar Eap som en bro mellan bakterien och värdens vävnad där kolonisation börjar. Detta bidrar till att sannolikheten att bli koloniserad eller infekterad av *S. aureus* ökar.

I den andra delen av avhandlingen har vi studerat inflytandet av Eap på vårt immunsystemet. I tidigare studier hade man redan visat att Eap kan binda till en molekyl som heter ICAM-1, en ytstruktur som finns på nästan alla celltyper. Denna molekyl har visat sig vara väldigt viktig i två sammanhang, först för att neutrofiler (försvarsceller) ska kunna binda till endotelceller och kunna ta sig från blodet till vävnaden där infektionen äger rum, och där kunna döda bakterierna. För det andra har ICAM-1 också visat sig ha en avgörande roll när främmande molekyler ska presenteras till vårt immunsystem. För att immunsystemet ska kunna känna igen något icke kroppseget, måste dessa främmande molekyler bli upplockade av presenterande celler (APCs= antigen presenting cells) och visas upp for mer specifika celler, framförallt T celler som därefter förökar sig och angriper och rensar bort bakterierna. Det är extremt viktigt att alla främmande bakteriella ämnen som kommer in i vår kropp blir ”riktigt” presenterade, annars kommer immunsystemet inte att känna igen dem och vi skulle få återkommande infektioner. Det är i den presentationsprocessen som ICAM-1 är så viktig.

Vi har kunnat visa att Eap binder till ICAM-1 och förhindrar både att neutrofiler tar sig genom endotelceller och att Eap hämmar T-cellers tillväxt. Denna hämning har visat sig vara koncentrationsbetingad, vid låga Eap koncentrationer har T-celler stimulerats och vid höga koncentrationer har T-celler dött. (**Publikation III och IV**). Det är intressant att påpeka att antikroppar mot Eap (fårantikroppar) kan förhindra bindning och internalisering av *S. aureus* in i värdceller. Humana antikroppar mot Eap kunde förhindra både den stimulerande och dödande effekten på T-celler. Man kan tänka sig att i framtiden skulle man kunna använda antikroppar mot Eap som vaccin, eller som behandling av *S. aureus* infektioner.

Vi kan sammanfatta våra resultat genom att påstå att Eap spelar en avgörande roll under en infections med *S. aureus*. Vi har kunnat visa att Eap från *S. aureus* har en

förmåga att binda, internalisera och modifiera immunförvarets genom att hämma T-cellers förökning. Allt detta kulminerar i att *S. aureus* genom att ha Eap får en rejäl försprång och kan etablera sig rätt så bra i värden innan immunsystemet överhuvudtaget hinner reagera och infektionen är ett faktum.

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