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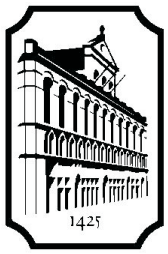
HUMAN ENDOTHELIAL CELLS, BACTERIA AND MONOCYTES IN ENDOCARDITIS: CELLULAR INTERACTION ANALYSIS IN VITRO



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ABBREVIATIONS

agr	accessory gene regulator
ClfA	clumping factor A
EC(s)	endothelial cell(s)
ECM	subendothelial extracellular matrix
F V(a)	(activated) coagulation factor V
Fg	fibrinogen
FIX	coagulation factor IX
Fn	fibronectin
FnBPA	fibronectin binding protein A
FnBPB	fibronectin binding protein B
FVII	coagulation factor VII
FX(a)	(activated) coagulation factor X
ICAM-1	intercellular adhesion molecule
IE	infective endocarditis
IL-	interleukin- (1, 6, 8, …)
L. lactis	*Lactococcus lactis*
MCP-1	monocyte chemoattractant protein 1
NBTE	non-bacterial thrombotic endocarditis
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
S. sanguis	*Streptococcus sanguis*
sar	staphylococcal accessory regulator
TF	tissue factor
TFA	tissue factor activity
TFPI	tissue factor pathway inhibitor
TNF-α	tumor necrosis factor α
VCAM-1	vascular adhesion molecule
vWbp	von Willebrand binding protein
vWf	von Willebrand factor

CHAPTER I

General Introduction

1 – Infective endocarditis

Definition and epidemiology of infective endocarditis

Infective endocarditis (IE) is an acute or subacute inflammatory reaction of the endocardial, inner surface structures of the heart, which leads to tissue destruction and possible occurrence of septic emboli (1-3). Bacterial infection is mostly seen on the heart valves, but neighbouring endothelial tissue as well as artificial patch material and arterio-venous shunt connections can be involved. The incidence of IE varies around 15 - 30 per 1 million inhabitants with only 5 % of the cases present at infant age (1, 4, 5). The majority of children developing endocarditis have an existing underlying congenital heart disease (2, 6).

Acute IE is caused by virulent microorganisms such as *Staphylococcus aureus* or *Streptococcus pneumoniae* which rapidly destroy the intact heart valve and lead to formation of vegetations and a disseminated infection (2, 3, 7). Vegetations are platelet-rich fibrin clots in which the microorganism and blood cells are embedded and as such are less accessible to host defence mechanisms (8).
Subacute endocarditis is characterized by a slow onset of disease which progresses over weeks and occurs mainly on pre-damaged heart valves (5, 7). Underlying bacterial microorganisms are less pathogenic as e.g. *Streptococcus viridans* or *Staphylococcus epidermidis*.

Diagnosis of endocarditis is still a clinical and diagnostic challenge. Unspecific clinical symptoms like fever and general malaise, preliminary antibiotic treatment and other associated conditions are responsible for obscuring diagnosis, which leads to a prolonged interval between onset of disease and diagnosis of up to 5 weeks (2, 5, 7).
Clinical diagnosis is based on the (modified) Duke criteria (9, 10). The Duke criteria rely upon major and minor criteria. Cases are classified as definite, possible or rejected based on clinical and pathological findings. Echocardiography plays a major role in the diagnosis of clinically suspected endocarditis with transoesophageal echocardiography having a positive predictive value of 90% (11). Disease severity

depends on systemic complications including septic emboli, which lead to serious infective and neurological complications. Due to difficulties in identification of the underlying pathology and severity of complications, IE is associated with a high lethality of up to 18% (1, 12). The pathogen is often difficult to eradicate and prolonged antibacterial treatment causes increased drug resistance.

2 - Bacterial contributing factors

Staphylococcus aureus

S. aureus is an opportunistic pathogen commonly associated with a wide range of acute and chronic infections, including IE (3, 13). The incidence of community acquired and hospital acquired infection is steadily increasing and treatment is hindered by occurrence of multidrug-resistance strains (3, 13).

S. aureus encounters for 25 - 35 % of all endocarditis cases causing a severe valvular destruction and associated systemic complications. Patients at risk include hospitalized patients, patients with prosthetic valves, elderly and intravenous drug abusers (1, 14-16). Clinically the infection is characterized by an acute onset with high fever, general malaise and frequent involvement of previously undamaged valves. Often, the diagnosis is made at an advanced stage, because specific symptoms are missing (7).

The ability of *S. aureus* to colonize host cells, in particular cells at the endovascular site, and to establish a local infection is facilitated by a variety of distinct bacterial surface proteins, collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). These molecules interact with high affinity to extracellular matrix (ECM) proteins adsorbed on host cells or present at sites of tissue injury. Well-characterized *S. aureus* MSCRAMMs are the fibronectin (Fn)-binding proteins (FnBPs) FnBPA and FnBPB and clumping factor A (ClfA) (13, 17-20). Investigations of clinical isolates showed a more severe cause of infections when strains expressed both, FnBPA and FnBPB surface molecules (21).

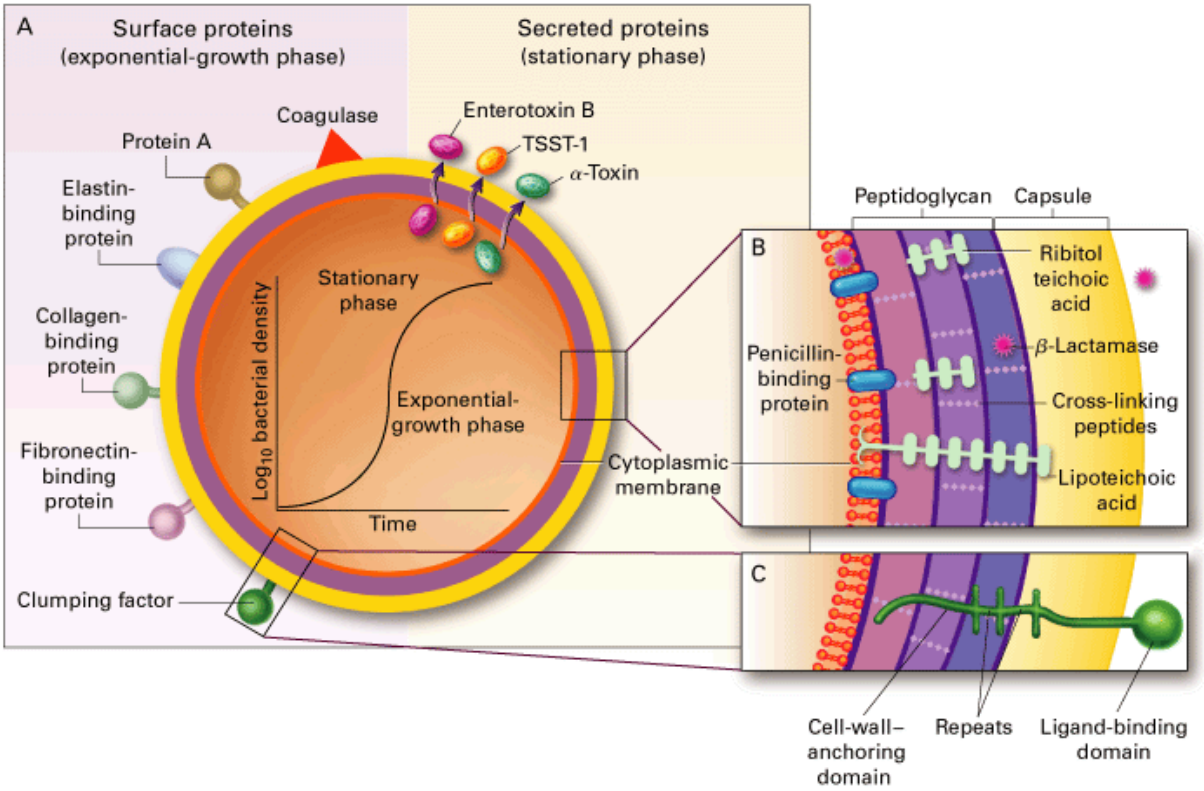

Figure 2: Surface structure of *S. aureus*
Panel A demonstrates the surface and secreted proteins. The growth phase shown in the graph influences the expression of many of these proteins which are mediated by regulatory genes such as *agr*. Panel B and C present the cell envelope. TSST-1 = toxic shock syndrome toxin1.
Lowy NEJM 1998 (13)

Both FnBP molecules efficiently bind Fn, and to a lesser extent fibrinogen (Fg) and elastin, and as such constitute a Fn-mediated molecular bridge between the bacterium and host cell Fn receptors, e.g. the $\alpha_5\beta_1$ integrins (22). *Staphylococcal* ClfA proteins bind Fg and in turn can activate platelets and enable *S. aureus* to aggregate at sites of endothelial lesions (18). In addition, ClfA may utilize Fg to interact directly with the integrin $\alpha_5\beta_1$ receptor on ECs. Via *S. aureus* collagen adhesin, encoded by *cna*, and protein A-von Willebrand factor (vWf) binding, capability of binding to collagen is ensured (23-25).

Peptidogycan is the main component of the cell wall of *S. aureus* and is suggested to have an endotoxin-like activity. It mediates the production of cytokines, complement activation and platelet aggregation (13, 26). Ribitol teichoic acids and lipoteichoic acids are other major components of the cell wall.

The expression of *S. aureus* virulence factors is paralleled by the global regulators “accessory gene regulator” (agr), “*staphylococcal* accessory regulator” (sar) and others (27-29). Expression of adhesins are coordinated during the exponential growth and secretion of soluble factors in the static growth phase (13). Recently, strains encoding Fn and Fg binders by definite genes did not coincide with infectivity in vivo (30). This exemplifies the complex interaction of mechanisms involved, differential gene expression, gene redundancy and host factors.

Upon interaction of *S. aureus* with human endothelial cells (ECs), a variety of proinflammatory endothelial responses are induced by cell surface expression of the cell adhesion proteins ICAM-1 (CD54) and VCAM-1 (CD106) mediating monocyte adhesion (31) and secretion of chemokines (e.g. IL-8, MCP-1) and proinflammatory cytokines (32). Moreover, *S. aureus* facilitates endothelial TF-antigen expression and subsequent TF-dependent coagulation which is synergistically enhanced by adhering blood monocytes, supporting formation of vegetations as mentioned before.

Furthermore, *S. aureus* plays an important role in inducing coagulation as well as in fibrinolysis. *Staphylococcal* coagulase is a potent activator of prothrombin which in turn converts fibrinogen in unsoluble fibrin (33). Also the von Willebrand-binding protein (vWbp) of *S. aureus* is known to mediate coagulase activity. In this way, vWbp might contribute to endothelial adhesion and fibrin production. Regenerated fibrin, activated thrombocytes and bacteria are main components of characteristic vegetations. Herewith the microorganism hides itself to host defence mechanisms and antibiotic therapy. The coagulation pathway via coagulase is an alternative pathway to evoke fibrin production and no physiological inhibitors are known for this process.

Staphylokinase activates plasminogen and forms a complex with plasmin. This complex needs to interact with fibrin to maintain its activity (34). Fibrinolysis and proteolysis of the extracellular matrix by plasmin are initiated. This process seems to be important in supporting the dissiminating manner of infection, but its contribution to the virulence of *S. aureus* is not fully understood.

The necessity of FnBPA and FnBPB for bacterial adherence to human ECs has been demonstrated by several groups (22, 35-38). In addition, expression of *S. aureus*

FnBPs conferred invasiveness to genetically distinct and otherwise non-invasive bacterial strains, such as *Lactococcus lactis* and *Staphylococcus carnosis* (39). Therefore this thesis focused on the role of FnBPA in bacterial adhesion and subsequent inflammatory and procoagulant response being important steps in the early pathogenesis of IE.

Staphylococcal **adhesin fibronectin binding protein A**

Staphylococcal surface proteins have certain structural components in common. The structural organization of *S. aureus* FnBPA is known from different studies and is outlined in figure 3.

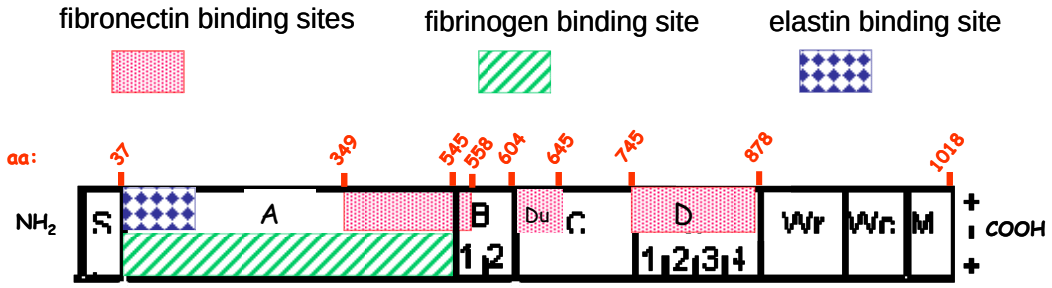

Figure 3: *Staphylococcal* FnBpA domains
Subdomains named A, B, C, D and Du. S is the leader sequence, Wr and Wc are wall anchoring components, M a membrane component (H. Beekhuizen, unpublished).

The FnBPA protein consists of 1018 amino acids divided into 4 general domains, termed A, B, C and D, flanked by a short N-terminal signal sequence and C-terminal sequences required for cell wall anchoring (17, 19, 40). The B domain consists of two amino acid repeats B1 and B2 which is linked to the C domain that encompasses region Du (see Fig. 3). The D region is characterized by three consecutive repeats, named D1, D2 and D3 that resemble Du, and one incomplete repeat (D4). The N-terminal A domain mediates binding to fibrinogen (Fg) (22) (41, 42) and elastin (42,

43), whereas the C- terminal region with domains B-DuC-D as well as the region encompassing the hinge between domain A and B (e.g. residues 432 to 559) bind fibronectin (Fn) (22, 44-46). Recently, this traditional organization was refined into further subdomains, which in parallel may contribute to binding (44, 47).

Facilitated by the binding sites to extracellular matrix components shown above, FnBPA mediates bacterial adhesion to human endothelium (20, 36, 48, 49). Since bacterial adhesion is essential to trigger diverse endothelial proinflammatory responses, the question arises if FnBPA is capable of subsequently inducing endothelial activation. A few reports support this suggestion and show that FnBPA plays a role in IL-6 production (50). Other authors have shown that FnBPA induces T-cell activation as well as platelet aggregation (51-53). In addition to these in vitro findings, *staphylococcal* FnBPA was shown to be essential for endovascular disease progression in vivo. Using a rat model of experimental endocarditis and a model of heterologous expression of *staphylococcal* adhesins in *Lactococcus lactis*, Que et al. demonstrated that the expression of FnBPA was a prerequisite for *S. aureus* to invade ECs and to establish a persistent and recurrent endovascular disease (48). *Staphylococcal* FnBPA is verified to mediate endothelial adhesion and seems also an important player mediating endothelial activation during *S. aureus* endovascular infections. These functional capacities of FnBPA and the described multi-domain molecular structure of this adhesion have directed our interest to specify the contributing role(s) of single FnBPA domains to EC activation and IE pathogenesis.

Investigation of individual staphylococcal surface molecules - *Lactococcus lactis* mutant strains

To enable investigations of the role of the individual *staphylococcal* surface proteins in IE, single molecules or combination of these were expressed by gene transfer in a surrogate bacterial background lacking the multiplicity and redundancy of the *S. aureus* strain (8, 39, 54). Therefore strains of the non-virulent *L. lactis* subspecies *cremoris 1363* were used (54). *L. lactis* does not express adhesins to mammal matrix proteins and more important, *staphylococci* and *lacotococci* share the mechanism of

processing their cell wall proteins by the LPXTG C-terminal motif to anchor the polypeptides to the peptidoglycan (54). This condition is necessary to facilitate expression of the *staphylococcal* proteins on the recipient bacterium. Genetic transfer made use of a newly constructed vector allowing the shuttling of cloned genes between *Escherichia coli* and *L. lactis* (39, 54). As a result, *L. lactis* mutants expressing one of the staphylococcal MSCRAMM FnBPA, FnBPB or ClfA on their surface were constructed by gene transfer of either the *fnbA*, *fnbB* or *clfA* gene of *S. aureus* (54). To allow selection, all recombinant *lactococci* were made resistant to erythromycin. In a further step, *L. lactis* mutants expressing only single parts of the FnBPA molecule, as describes above, were built to investigate single (sub)domains of the *staphylococcal* FnBPA molecule. Bacterial phenotype and functional characteristics of these mutant strains were confirmed by adhesion assays to Fg and Fn as well as by invasion to ECs (20, 39, 48, 54).

Streptococcus sanguis

Viridans streptococci are one of the main causative species found in subacute endocarditis developing on pre-damaged endothelium (8, 55). These bacteria are identified in about 26 % of all adult cases (56) and are found as common causative organisms in children accounting for 20 - 47 % of the endocarditis cases (2, 7, 55). Onset of disease is characterized by a protracted course including low-grade fever and unspecific clinical symptoms like night sweat and weight loss (7). Cutaneous manifestations and embolic complications are less common than in acute IE. *S. sanguis* belonging to this group of bacteria is permanently present in the oral flora (57). Most human plasma components and ECM proteins serve as binding sites for *viridans streptococci*. They are known to bind to mucins and salvatory proteins. *S. sanguis* expresses surface polysaccharides named glucans which are produced by glycosyltransferase (gft) and fructosyltransferase (ftf) enzymes (58). Glucan production mediates *streptococcal* adhesion to fibrin clots in vitro (59). Several other adherence factors orchestrate *streptococcal* adherence. Extracellular matrix adhesins like Fn-binding proteins (e.g. FBP-130) were identified and its role in

enhancing severity of IE was demonstrated in an animal model (60, 61). In addition, FimA as an oral mucosal adhesin mediates bacterial attachment (62).

Viridans streptococci are potent in binding to ECs and enable an endothelial inflammatory activation leading to endothelial adhesion molecule expression (ICAM-1, VCAM-1, E-selectin), which is mediated by the surface protein I/II (32, 63-65). Soluble protein I/II has been shown to evoke the same endothelial reaction and therefore it might pre-condition the endothelium to be more susceptible for bacterial adhesion (63). In addition, cytokine production of IL-1, IL-6 and IL-8 is facilitated by ECs and monocytes upon infection with *viridans streptococci.*

Additively, IL-1 was found to induce an increased susceptibility for IE in animals (66-69). About 60 % of *streptococci* serve as a prothrombotic trigger by inducing monocyte activation and platelet aggregation in vitro and in vivo, this in addition to their role in building the endocardial vegetation (70-73). *Streptococcal* platelet aggregating factors (phase I and II antigens, pblA, pblB, pblT) are important players (8). Moreover, cross-reactive immunodeterminants on the surface of *S. sanguis* and collagen-I promote platelet aggregation (74). Immunoglobulin G mediated adhesion of *streptococci* to platelet membrane glycoprotein and a platelet aggregation-associated protein (PAAP) expressed by *S. sanguis* were found to contribute to the thrombogenic potential of this microorganism (70). Furthermore, *S. sanguis* was found to facilitate induction of tissue factor mediated coagulation response being the major procoagulant pathway in IE (73, 75, 76). To ensure persistent bacteremia *streptococci* inherit different mechanisms to survive despite host defence mechanisms. An autolysin, AtlA, as well as resistance to platelet bactericidal factors were shown to contribute to bacterial survival in the bloodstream supporting bacterial virulence (77, 78)

Staphylococcus epidermidis

S. epidermidis belonging to the group of coagulase-negative *staphylococci* (CNS) is part of the normal human skin flora and found on mucous membranes. An increasing important role as a pathogen has been recently recognized with CNS accounting for

30 % of all nosocomial bloodstream infections including a high percentage of 55 – 75 % methicillin resistant strains in these nosocomial isolates (79).

S. epidermidis strains are typically found in about 30 % of adult patients presenting with prosthetic valve endocarditis and a minor group of cases presenting with acute native valve endocarditis (8 %) (80). Also in the paediatric age group, *S. epidermidis* is frequently associated with infected prosthetic material, representing about 8 % of the infant cases (2, 7).

The clinical cause in subacute prosthetic valve endocarditis is demonstrated by unspecific clinical symptoms of infection and malaise as mentioned earlier for *viridans streptococci*. Clinical and epidemiological data suggest that the strains causing acute and subacute valve infection are different *S. epidermidis* populations (80). This was verified by genotyping clinical isolates and investigation of those in a worm model, which showed a more virulent subset of strains observed in patients with native valve endocarditis (80). As said, the vast majority of infections attributed to *S. epidermidis* originate from nosocomial catheter infections (3, 79-81).

Virulence of *S. epidermidis* is essentially related to its capacity of biofilm formation which enables adhesion and colonization of different kinds of surfaces as cellular membranes, ECM and artificial devices (82, 83). Several surface proteins involved in biofilm formation have been identified, but the complex underlying pathogenic mechanisms of *S. epidermidis* infections are not fully understood yet (81). Most clinical isolates of *S. epidermidis* strains are able to produce a biofilm (82) which is formed in two steps: Surface proteins and antigen capsular polysaccharide adhesin (PS/A) enable immediate bacterial attachment (81). Further bacterial accumulation is mediated by an accumulation-associated protein as well as a capsular polysaccharide intercellular adhesin (PIA) (81, 84). Animal studies showed the role of PS/A and PIA in catheter related infections and endocarditis (81, 85). Polysaccharide adhesins are encoded by the *ica* gene cluster, which appears to be more frequent in clinical isolates than in skin flora (81, 84). *S. epidermidis* surface proteins mediate binding to Fg, Fn and vitronectin and as such act as further virulence factors, most of them containing the LPXTG motif (86). Different groups identified a Fg-binding protein Fbe (87, 88) and SdeG (89, 90), an Fn-binding protein (pSE109FN) (91), and collagen-I binding sites (SdrF) (92) which were confirmed to play a role in catheter

associated infections. The surface protein accounting for fibrinogen binding was found to be in part homologous to the *S. aureus* clumping factor (93).
The induced inflammatory and procoagulant reaction affects patient outcome. Endothelial cells are activated by *S. epidermidis* and express the cell adhesion molecules ICAM-1 and VCAM-1 (75, 94). Upon contact with *S. epidermidis,* a variety of cytokines like IL-1, IL6 and TNF-α are produced by monocytes (95, 96).
S. epidermidis was shown to induce a very low endothelial procoagulant activity but enables an increased TF-mediated procoagulant activity after contact with monocytes (32, 75). Epidemiologic and clinical observations as well as in vitro studies show growing evidence that *S. epidermidis* is a more relevant pathogen than previously thought.

3 - Pathogenesis of IE

In IE, bacterial metastasis involves the preferential interaction of disseminating bacteria with cardiac vascular ECs. Essential for the infection process is the propensity of the bacteria to colonize endovascular tissue, allowing the pathogens to adhere and invade ECs and spread via the bloodstream to other tissues (3, 13).
Based on a first postulated model on the pathogenesis of endocarditis by Angrist en Oka (97), further research by different groups has given insight in the complex mechanisms in IE, leading to a model, characterized by the following main steps: bacterial colonization of valvular endothelium, inflammatory and procoagulant responses leading to formation of vegetations and persistence of disease implicating tissue invasion and bacterial dissemination. A model of invasive *S. aureus* infections was summarized by Lowy et al. as shown in figure 1 (13).

Host factors - Human vascular endothelial cells

ECs cover the luminal surface of blood vessels, constructing a monolayer of cells, which represents the interface between tissue and blood. The endothelium functions as a barrier and allows transport of metabolites and leucocytes between the two facing compartments. Furthermore, it plays a role in the maintenance of the vascular

tone and interacts with vasoactive hormones. The intact endothelium is resistant to bacterial colonization and thrombus formation (98).

In IE, the endothelium functions as the primary site of the infection process, when it loses its integrity at the very early onset of disease (97). Endothelial lesions originating from pre-existing valve abnormalities lead to subsequent exposure of the underlying ECM proteins. The primary wound healing process implicates tissue factor mediated coagulation and deposition of fibrin and platelets. The so called non-bacterial thrombotic endocarditis (NBTE) is a preferred target for bacterial adhesion in patients with bacteremia (8).

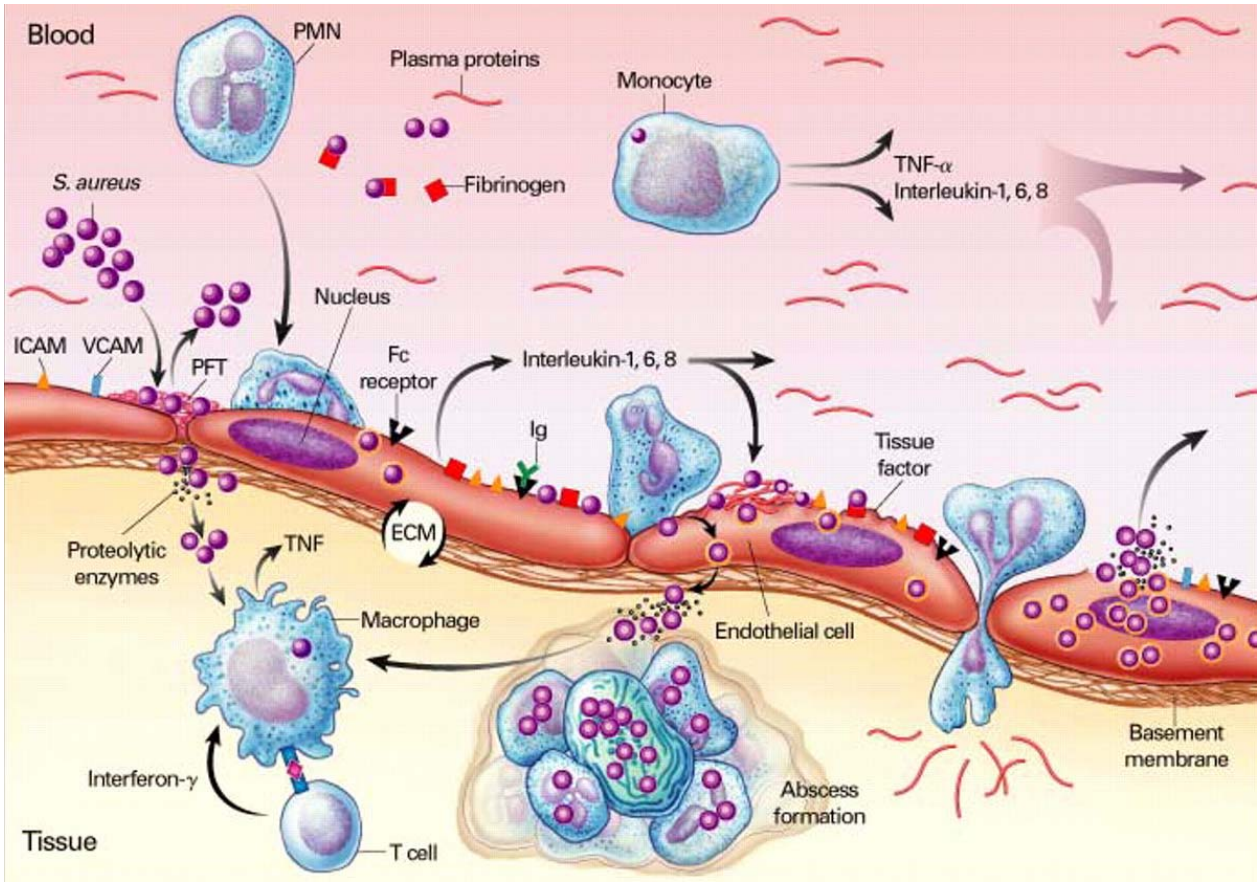

Figure 1: Pathogenesis of *staphylococcal* invasive infection
Starting from the left side of the panel, *S. aureus* binds to ECs or platelet-fibrin thrombi (PFT) which have formed at the endovascular site. After phagocytosis by ECs, bacterial proteolytic enzymes enable *S. aureus* to disseminate in the bloodstream and adjacent tissue. Fibrin deposition and formation of the vegetation is initiated by TF expression. Endothelial activation includes intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) expression and cytokine production of IL-1, 6 and 8. Leucocytes subsequently adhere to ECs. Cytokine production of IL-1, 6 and 8 as well as of tumor necrosis factor α (TNF-α) and TF expression by monocytes and macrophages is enhanced, which contributes to the manifestation of invasive endovascular and systemic disease. PNM = polymorphonuclear leucocyte. Further explanation is referred to in the text (13).

Alternatively, IE can occur on intact endothelium when highly pathogenic bacteria like *S. aureus* adhere to ECs, triggering primary endothelial damage (8, 13).
The process of bacterial adhesion and invasion to the vascular endothelium is central in disease onset. Recent studies emphasize the significant contribution of bacterial surface molecules to EC adhesion, collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) and demonstrate their role in inducing a variety of proinflammatory endothelial responses resulting in leucocyte accumulation, cell damage and fibrin deposition (8, 36, 88, 99, 100). Dependent on the pathogenicity of the microorganisms either cytotoxic endothelial damage or endothelial cell activation occurs.

Proinflammatory response

Monocytes are important players in the inflammatory body reaction. They are part of the specific and non-specific immunological systems. Being produced in the bone marrow, they circulate in the blood for a few days. After adherence to the endothelium, monocytes migrate to the underlying tissue and differentiate into macrophages. They are able to phagocytose microorganisms.
When activated, the endothelial cell inherits an important role in the progress of endovascular infection. Upon interaction with bacteria, a variety of proinflammatory endothelial responses are induced, by cell surface expression of the cell adhesion proteins intercellular adhesion molecule 1 (ICAM-1; CD54) and vascular cell adhesion molecule 1 (VCAM-1; CD106), mediating monocyte adhesion (31, 75). Cytokines and chemokines like IL-1, IL-8, MCP-1 are secreted by ECs and monocytes (8, 32, 66, 69, 75, 95).

Procoagulant response and thrombus formation

Following valve colonization, microorganisms overrule host defence mechanisms and persist at the infected site, where they contribute to disease progression. This seems via formation of vegetation, in which bacteria are enveloped.

Activation of the coagulation system is a major key pathway in IE. It accounts for the formation and maintenance of the endocardial vegetation. Both, bacterial and host factors orchestrate tissue factor (TF) production, which in turn initiates coagulation via the extrinsic coagulation pathway, as well as platelet aggregation.

The extrinsic coagulation pathway is dependent on TF, a 45 kDa membrane bound glycoprotein (101). The molecule consists of an extracellular domain of 219 amino acids, a transmembrane component of 23 amino acids and a cytoplasmatic domain of 21 amino acids. To acquire procoagulant activity, TF forms a calcium-mediated complex with coagulation factor VIIa (FVIIa), capable of directly activating FX to FXa. The TF/FVIIa complex also activates coagulation factor IX (FIX) to FIXa, which subsequently activates factor X (FX).

The activated form of FX (FXa) is part of the prothrombinase complex that consists of activated factor V (FVa), FXa, calcium ions, phospholipids and prothrombin. This complex cleaves prothrombin into thrombin, which in turn converts fibrinogen into fibrin.

The process that leads to the activation of the clotting system on the endothelial surface upon bacterial challenge has been addressed earlier (32, 75, 102).

TF is synthesized and released by monocytes when they bind to fibrin clots, a process, which is significantly increased in vegetations formed in experimental IE upon stimulation with *S. aureus, S. sanguis* and *S. epidermidis* (73, 94, 102). Since TF is a cell membrane-associated protein, ECs, monocytes and fibroblasts can all account for the production of TF at the infected site.

TF is not expressed on the cell surface of healthy, non-infected ECs, but cytokines and bacterial factors (e.g. lipopolysaccharides) are known to potentiate TF expression (26, 76, 101). Circulating monocytes constitutively release low amounts of TF into the circulation, but upon their activation by lipopolysaccharides, monocytes rapidly upregulate their TF expression.

Under infective conditions in vitro, *S. aureus* induced endothelial derived TF-antigen expression and subsequent TF-dependent coagulation, in contrast to *S. sanguis* and *S. epidermidis* (32). Whether ECs contribute to TF synthesis in vivo is still controversial.

IL-1 is known as a potent TF activator in ECs in vitro. Interestingly, the *S. aureus* stimulated TF activity (TFA) could not be abrogated by an IL-1 receptor antagonist, suggesting that IL-1 and *S. aureus* enable different TF synthesis pathways (75). Importantly, this procoagulant process is synergistically enhanced by monocytes which potently facilitate TFA, also upon *S. sanguis* and *S. epidermidis* stimulation in vitro and in vivo (75, 102, 103). Therefore, bacteria enable monocytes to produce TF and enhance the procoagulant state. The TF activation process is not fully understood yet, thus bacterial surface molecules as well as ECM molecules like Fn and cell adhesion molecules (ICAM-1 and VCAM-1) seem to be required (73, 75, 103). In contrast, monocytes are involved in the downregulation of TFA by inducing IL-10 production via TNF-α (104, 105). Even more, monocytes are capable to directly regulate TFA by production of tissue factor pathway inhibitor (TFPI) when adherent to Fn (106). This supports the important role of monocytes as regulative players, especially in a context where extracellular matrix proteins are involved.

Thus, TFA contributes to formation and maintenance of the vegetation which parallels bacterial persistence at the endovascular site causing severe dysfunction of the heart valve. As such, it is considered a critical primary feature in the early pathogenesis of IE (3, 8).

Bacterial induced platelet aggregation plays an important role in the formation of vegetations, with activated platelets further facilitating coagulation reactions on their negatively charged surface. On the opposite side, platelets release anti-microbial peptides and inflammatory mediators, which are part of the anti-infective host defence mechanisms. The investigation of platelet interactions and their role in IE was beyond the scope of this thesis.

4 – Tissue engineering in heart valves

Congenital and acquired heart valve diseases are a major cause of morbidity and mortality. Novel therapeutic strategies have improved the quality of life in patients with artificial or biological heart valves, but treatment associated limitations and complications are still of major concern. Bleeding due to required anticoagulation,

limited durability and a higher risk to develop endocarditis are among the main complications (107, 108). A significant limitation is also the inability of current replacement valves to grow in the pediatric patients resulting in repeated surgical interventions.

To minimize associated risk factors and increase the biocompatibility after heart valve implantation are major concerns in research, directing research efforts in this area.

Autologous matrix-based tissue engineered heart valves are under investigation as patient-derived valve replacements. The patient derived matrix scaffold implicates a potential to implant a completely autologous heart valve with possible tissue remodelling, expected to lead to an increased biocompatibility and durability as well as an expected lower risk of developing endocarditis (109-112). A tissue-engineered valve is expected to respond to growth and physiological forces similar to a native valve (113).

Different methods and materials are under investigation to serve as a basic matrix. Two main approaches have been made so far. First, bioresorbable synthetic scaffolds are used, which, by regeneration, are expected to remodel in vivo to form a functional valve composed of patients cells and connective tissue proteins (113, 114). For this purpose, scaffolds composed of synthetic materials (e.g. polylactic acid (PLA), poly4-hydroxybutyrate (P4HB), polyglycolic acid (PGA)), hydrogels or natural polymers (e.g. collagen, fibrin, elastin) have been seeded with autologous cells (115). Second, decellularized xenogenic tissues are investigated leaving an intact, mechanically functional tissue matrix followed by cell seeding in vitro or in vivo (113, 114). Xenogenic tissues are decellularized because the antigenicity is assumed to originate from the cellular structures (113).

Advances in structure and mechanical durability of these constructed valve conduits were made by different research groups (110, 111, 116-119). Biodegradable matrices are suggested to serve as a matrix, which leaves the potential to grow in the paediatric patient group. Current research in tissue engineering of heart valves focuses on testing the material for biocompatibility and hemodynamic features (116, 120-122).

Cells, to be seeded are an important component of the tissue-engineered heart valve. Different cells have been used with a focus on endothelial cells, endothelial

progenitor cells and mesenchymal stem cells (123). Another point of interest is whether matrices should be pre-seeded in vitro or rather let them be seeded after implantation in vivo (124).

Unfortunately, these principals were expected to be much more promising, and major limitations were shown in animal experiments (110, 116). Studies demonstrate that these materials have a number of technical and constructional drawbacks. Residual cells of decellularized scaffolds can serve as an immunological trigger and synthetic polymers lack natural attachment points. Overshooting fibrosis resulted in leaflet retraction and valve incompetence (114).

The ideal tissue engineered heart valve may prove to be a completely autologous derivate, developed entirely from materials isolated from the patient in question. Fibrin, a major structural protein involved in the wound healing process, represents a potentially ideal cell delivery vehicle for the synthesis of completely autologous cell-seeded structures because of its routine isolation from the patient's blood sample (125). Therefore, a fibrin-based valve serving as a basic matrix is under investigation (120, 121, 126). This fibrin-based tissue engineered heart valve showed encouraging results in a sheep model with good tissue remodelling (125).

In the same context and in addition serving as a biodegradable scaffold, collagen based matrices are under investigation (127, 128). This approach is based on the fact that cells entrapped in collagen gels contract and compact the gel which forms a highly aligned construct upon mechanical stimulation (113). By these steps, a mold for valve leaflets could be formed (129).

Fibrin and collagen are known players in different inflammatory and procoagulant pathways, especially known as a potent trigger of thrombosis. The interaction of these matrices with *S. aureus* and other bacteria is not known yet, but the potential role of these newly constructed heart valves, and especially their matrix, in the pathogenesis of endovascular infection seems important.

CHAPTER II

General hypothesis and specific aims of the project

Surface molecules of *S. aureus* are involved in the colonization of vascular endothelium being a crucial primary event in the pathogenesis of infective endocarditis. The ability of these molecules to also launch an endothelial proinflammatory and procoagulant response, that characterize IE, is not known.
This endothelial response might change when ECs are seeded on different matrices used in tissue engineering.

The aim of this thesis was to demonstrate the individual role of three well-known *S. aureus* surface molecules, fibronectin-binding protein A (FnBPA) and B (FnBPB) and clumping factor A (ClfA) in the early pathogenesis of endocarditis.
Their capacity to promote bacterial adherence to cultured human ECs and to induce proinflammatory and procoagulant responses in ECs were investigated by using the non-invasive surrogate bacterium *L. lactis*, which by gene transfer expressed *staphylococcal* FnBPA, FnBPB or ClfA molecules.
Suggesting that the Fn-binding plays a central role we further questioned whether these bacterial - EC interactions could be reproduced by single or combined FnBPA (sub)domains (A, B, C or D). Using a large library of *L. lactis* expressing single different FnBPA (sub)domains or combinations thereof, we wanted to evaluate the role of the distinct domains with Fn-binding activities to provoke the typical endothelial proinflammatory and procoagulant responses observed during the early phase of *S. aureus* endovascular infections.
In detail the endothelial proinflammatory phenotype is evaluated by ICAM-1 and VCAM-1 surface expression as well as by cytokine production. Procoagulant activity is investigated by measuring endothelial derived tissue factor activity (FXa assay) as well as monocyte mediated enhancement of procoagulant activity.

Furthermore, based on the knowledge of earlier studies (31, 32, 75), we suggest, that matrix bound ECs might express an alternated inflammatory and procoagulant phenotype. To identify the influence of matrices used in tissue engineering on bacterial - endothelial interaction upon stimulation with *S. aureus*, *S. sanguis* or *S. epidermidis*, we aim to transfer our experiments to ECs seeded on a fibrin and collagen gel matrix. This might state the contribution of matrices to induce pathways of evoking inflammation and fibrin deposition at the infected endovascular site.

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CHAPTER III

Fibronectin-binding proteins and clumping factor A in *Staphylococcus aureus* experimental endocarditis: FnBPA is sufficient to elicit procoagulant and inflammatory responses in human endothelial cells

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ABSTRACT

Surface molecules of *Staphylococcus aureus* are involved in the colonization of vascular endothelium being a crucial primary event in the pathogenesis of infective endocarditis (IE). The ability of these molecules to also launch endothelial procoagulant and proinflammatory responses, that characterize IE, is not known. In the present study we investigate the individual capacities of three prominent *S. aureus* surface molecules; fibronectin-binding protein A (FnBPA) and B (FnBPB) and clumping factor A (ClfA), to promote bacterial adherence to cultured human endothelial cells (ECs) and to activate phenotypic and functional changes in these ECs. Non-invasive surrogate bacterium *Lactococcus lactis*, which, by gene transfer, expressed staphylococcal FnBPA, FnBPB or ClfA molecules were used. Infection of ECs increased 50-100 folds with FnBPA- or FnBPB-positive recombinant lactococci. This coincided with EC activation, IL-8 secretion and surface expression of ICAM-1 and VCAM-1 and concomitant monocyte adhesion. Infection with ClfA-positive lactococci did not activate EC. FnBPA-positive *L. lactis* also induced a prominent tissue factor-dependent endothelial coagulation response that was intensified by cell-bound monocytes. Thus *S. aureus* FnBPs, but not ClfA, confer invasiveness and pathogenicity to non-pathogenic *L. lactis* organisms indicating that bacterium-EC interactions mediated by these adhesins are sufficient to evoke inflammation as well as procoagulant activity at infected endovascular sites.

INTRODUCTION

Staphylococcus aureus is a major cause of severe endovascular complications such as infective endocarditis (IE) (1, 2). In IE bacterial metastasis, in particular in patients without pre-existing injured heart valves, involves the preferential interaction of disseminating *S. aureus* with (cardiac) vascular endothelial cells (ECs). The vegetation, a thrombus consisting of clotted fibrin, blood cells and bacteria that forms on this infected/injured endothelial site, causes severe dysfunction of the heart valves and as such is seen as a critical primary feature in the early pathogenesis of IE (2).

The ability of *S. aureus* to colonize host cells, in particular cells at endovascular sites, and to establish a local infection is facilitated by a variety of distinct bacterial surface proteins, collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). These molecules interact with high affinity to extracellular matrix proteins absorbed on host cells or present at sites of tissue injury (3). Well-characterized *S. aureus* MSCRAMMs are the fibronectin (Fn)-binding proteins (FnBPs) FnBPA and FnBPB (4-6) and clumping factor A (ClfA) (7, 8). Both FnBP molecules efficiently bind Fn, and to a lesser extent fibrinogen (Fg) and elastin, and as such constitute a Fn-mediated molecular bridge between the bacterium and host cell Fn receptors, e.g. the $\alpha_5\beta_1$ integrins (4, 9-13). Staphylococcal ClfA proteins bind Fg and in turn can activate platelets and enable *S. aureus* to aggregate at sites of endothelial lesions (7, 14, 15). In addition, ClfA may utilize Fg to interact directly with the integrin $\alpha_5\beta_1$ receptor on ECs (16).

The necessity of FnBPA and FnBPB for bacterial adherence and invasion of human ECs has been demonstrated by several groups (reviewed in reference (17)). Deletion of these FnBPs from *S. aureus* was shown to be associated with a poorly adhesive, non-invasive phenotype (11, 13). In addition, expression of *S. aureus* FnBPs conferred invasiveness to genetically distinct and otherwise non-invasive bacterial strains, such as *Lactococcus lactis* and *Staphylococcus carnosis* (5, 18). Using live cell imaging, Schröder et al. recently demonstrated the capacity of staphylococcal FnBPA to induce centripetal bacterial movements on the endothelial cell surface and to delay bacterial phagocytosis (19). In a rat model of experimental endocarditis it

was confirmed that infection with *L. lactis* recombinants expressing FnBPA, in contrast to ClfA-expressing recombinants, resulted in persistent valve colonization and endothelial invasion with clinical symptoms resembling an *S. aureus* infection (20).

Previous studies including our own emphasized on the active contribution of vascular ECs to explain early stages in the pathogenesis of endovascular *S. aureus* infections, such as IE (21-24). It was shown that *S. aureus* organisms upon interaction with human ECs powerfully induced a variety of proinflammatory endothelial responses by cell surface expression of the cell adhesion proteins ICAM-1 (CD54) and VCAM-1 (CD106) mediating monocyte adhesion (23, 24) and secretion of chemokines (e.g. IL-8, MCP-1) and proinflammatory cytokines (23, 25, 26). Moreover, these infections with *S. aureus* initiated endothelial TF-antigen expression and subsequent TF-dependent coagulation which was synergistically enhanced by adhering blood monocytes (21, 24, 27).
Although the adhesive and invasive abilities of *S. aureus* are considered crucial for eliciting such endothelial activation as yet no effort has been put on elucidating this assumption at a molecular level. In the present study we therefore compare the individual capacities of three major *S. aureus* MSCRAMMs, FnBPA, FnBPB and ClfA, to modify the EC phenotype. We show that in particular the sole interaction of FnBPA molecules expressed in *L. lactis* is capable and sufficient to elicit proinflammatory and procoagulant activities in ECs. These activities are further amplified by adhering monocytes and as such typify early processes in the pathogenesis of IE.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Recombinant strains of the non-virulent *L. lactis* subsp. *cremoris 1363* expressing one of the staphylococcal MSCRAMM FnBPA, FnBPB or ClfA, on their surface were constructed by gene transfer of either the *fnbA*, *fnbB* or *clfA* gene of *S. aureus*, as described elsewhere (28). All recombinant *lactococci* were resistant to erythromycin to allow selection. *L. lactis* pIL253 was used as the control mutant strain and

expressed only the erythromycin-resistance determinant. A detailed enumeration of the bacterial phenotype and functional characteristics has been reported by Que et al. (18, 20, 28). All bacterial strains were prepared and kindly provided by Dr. P. Moreillon and were stored at -70°C in liquid M17 medium, i.e. M17 broth (Oxoid, Basingstoke, UK) plus 0.5 % glucose (Brunschwig Chemie, Amsterdam, The Netherlands) and 5 µg/ml erythromycin (Abbott B.V., Hoofddorp, The Netherlands), supplemented with 10 % (vol/vol) glycerol. For use in the infection assays bacteria were grown overnight at 30°C without shaking in M17 medium or on M17 agar plates supplemented with 0.5 % glucose and 5 µg/ml erythromycin, then opsonized for 30 min at 4 rpm in M199 containing 0.1 % (w/v) gelatin and 10 % fresh human serum and diluted to the appropriate concentration in medium 199 (M199; GIBCO Laboratories, Grand Island, NY) containing 5 % heat inactivated (30 min, 56°C) fetal calf serum (FCSi), 5 % heat inactivated human serum (HuSi) and 5 µg/ml erythromycin.

Bacterial adherence to human fibronectin

The recombinant *L. lactis* strains were tested for their ability to bind immobilized purified human fibronectin (Sigma, Poole, UK), using a 96-well microtitre plate assay and staining with Crystal violet, as described previously (9, 29). Bacterial adherence to fibronectin was expressed as the absorbance measured at OD_{570} by a Victor^2 multilabel counter using Wallac 1420 software version 3.0 (PerkinElmer, Turku, Finland) after subtraction of background values.

Preparation of EC and monocyte cultures

ECs were isolated from human umbilical veins from healthy donors and cultured on gelatin-coated tissue culture dishes in culture medium consisting of M199 supplemented with 0.1 mg/ml EC growth factor, 5 % HuSi, 5 % FCSi, 5 U/ml heparin and 1 mM L-glutamin, 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 100 U/ml amphothericin-B in a 5 % CO_2 incubator at 37°C, exactly as described elsewhere (23). Monolayers of second-passage ECs grown on gelatin-coated tissue plates or on gelatin-coated glass coverslips in 24-well tissue plates, yielding a minimal cell density of 800 ECs /mm^2, were used in the experiments. The THP-1 human monocytic cell line was maintained in RPMI 1640 containing 100 U/ml penicillin G, 0.1 mg/ml

streptomycin and 10 % FCSi. We showed previously that THP-1 cells expressed the surface adhesion molecules required for monocyte-EC interaction and produced TFA on appropriate stimulation (21, 24). Prior to their use in the experiments THP-1 cells were resuspended in M199 containing 5 % HuSi, 5 % FCSi and 5 µg/ml erythromycin. In the text THP-1 cells are mentioned as monocytes.

EC infection assay

Confluent monolayers of ECs were incubated under variable experimental conditions with opsonized *L. lactis* bacteria at 37°C, essentially as described for *S. aureus* bacteria (23). After this, the monolayers were washed with warm PBS to remove the bacteria that were not associated with the ECs. The percentage of infected ECs, being cells with at least one extracellular cell membrane-associated or intracellular bacterium, as well as the number of cell-associated lactococci per cell was determined under a light microscope, as described for infection with *S. aureus* elsewhere (22). The number of bacteria used in the infection assay was confirmed afterwards by colony counts after plating serial dilutions on M17 agar plates and overnight incubation at 30°C.

Flow-cytometric analysis of EC surface molecules

EC monolayers were infected for 1 h with variable numbers of opsonized recombinant *L. lactis* bacteria, washed with PBS and cultured for an additional period of 23 h. The cells were harvested using trypsin, washed and collected in cold PBS with 0.1% FCSi (wash buffer) and taken under three incubation steps on ice: 15 min with PBS containing 1% goat serum, 30 min with 1µg/ml of the appropriate mAb and 30 min with phycoerythrin-conjugated goat-anti-mouse Ig (Southern Biotechnology Associates Inc. Birmingham, Ala.). In between each step cells were washed with cold wash buffer. At least 5000 cells were analyzed by flow cytometry (Becton Dickinson FACSCaliber) using CellQuest® software. The following mouse mAb against surface molecules on human ECs or monocytes were used: anti-CD54 (ICAM-1) mAb 15.2 (Santa Cruz Biotechnology, Santa Cruz, Ca.); anti-CD106 (VCAM-1) mAb 1G11B1 (Biosource International, Camarillo, Ca.) anti-CD142 (TF) mAb HTF-1 (Becton Dickinson).

Determination of cytokines

Supernatants from ECs infected with recombinant *L. lactis* bacteria were assayed for production of the proinflammatory cytokines IL-6 and IL-8 and the anti-inflammatory cytokine IL-10 using the PeliKine Compact ELISA kit for human IL-8 (Sanquin, Amsterdam, The Netherlands) and Cytoset^{TM} immunoassay kits for human IL-6 and human IL-10 (Biosource International). The assays were performed according to the supplier's instructions. Limits for detection of IL-8, IL-6 and IL-10 were 8.0 pg/ml, 50.0 pg/ml, and 10.0 pg/ml, respectively.

Monocyte-EC adhesion and coculture conditions

About 1.5 x 10^5 monocytes in M199 containing 5% FCSi, 5% HuSi and 5 mg/ml erythromycin were added to monolayers of approximately 2.0 x 10^5 bacteria-infected ECs grown on gelatin-coated glass coverslips in 24-well cell culture plates and subsequently cocultured for 1 or 6 h at 37 °C in a 5 % CO_2 incubator. The 1 h time point was included because it is an optimal time point to determine immediate effects of EC activation on monocyte adhesion (23, 30), the 6 h time point being optimal for determination of the total TFA in these cocultures (24). After coculture the glass coverslips were washed 5 times with warm PBS to remove non-adherent monocytes. The remaining cells were fixed with methanol for 15 minutes and stained with Giemsa. The number of adherent monocytes was counted under a light microscope according our standardized method (30). In order to evaluate the contribution of cell surface adhesion molecules involved in monocyte-EC interaction to TFA in the cocultures, a set of experiments were performed in the presence of 10 µg/ml of anti-CD18 mAb (clone IB4) plus 10 µg/ml of anti-CD49d mAb (clone 15A8) (Central Laboratory for Bloodtransfusion, Amsterdam, The Netherlands).

RNA extraction and reverse transcriptase (RT) PCR for TF gene expression

Total RNA of infected ECs was extracted with RNAzol (Campro Scientific, Veenendaal, the Netherlands) according to the supplier's manual. cDNA preparation and subsequent PCR amplification was carried out exactly as described by us elsewhere (22). Primers obtained from GIBCO were used for human TF gene (sense, 5´-ATGGAGACCCCTGCCTGG-3´ and antisense, 5´-CCAGCAGAACCGGTGCTC-3´) and the housekeeping gene GAPDH (sense, 5´-CATCACCATCTTCCAGGAGC-

3´ and antisense, 5´-GGATGATGTTCTGGAGAGCC-3´). PCR products were analyzed by electrophoresis through 2 % agarose gels and were visualized with ethidium bromide.

Analysis of TF-dependent procoagulant activity (TFA)

Following infection and/or coculture with monocytes, TFA of EC monolayers was measured by the transformation of clotting factor X (FX) to FXa, using PefachromeFXa® (Kordia , Leiden, The Netherlands) as the chromogenic substrate of FXa, as has been described in detail elsewhere (31). FXa concentrations were calculated using a calibration curve of purified FX that was fully activated with Rusel Viper Venom (Chromogenix, Mölndal, Sweden). Values are given as mU FXa / well containing about 2 x 10^5 ECs and, in some experiments, variable numbers of EC-bound monocytes.

Statistical analysis

Data were analyzed with the SPSS program for Windows software, version 12 (SPSS GmbH Software, Muenchen, Germany). The paired Student's *t* test (two-tailed) was used to control for variation in measurements due to the fact that for each experiment ECs isolated from different donors were used. Results are expressed by the mean value ± SEM. Values of $p \leq 0.05$ were considered significant. The correlation between the percentage infected ECs and the number of bacteria used in the infection assay was assessed by nonlinear regression analysis.

RESULTS

Infection of ECs with recombinant lactococci expressing staphylococcal MSCRAMMs

First, the *L. lactis* recombinant strains were compared for their adhesion to immobilised human fibronectin. The data depict the fibronectin-binding phenotypes of lactococci expressing *S. aureus* FnBPA or FnBPB (Fig.1), in accordance with earlier publications (9, 18).

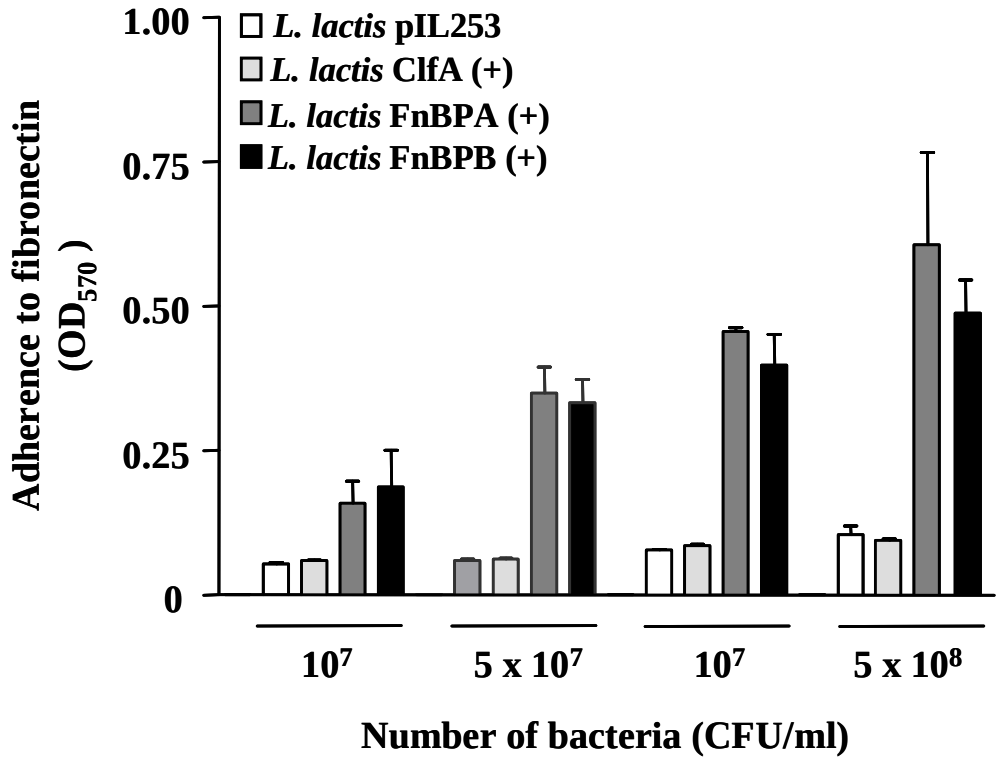

Figure 1: Adherence of *L. lactis* recombinants to human fibronectin.
Adherence of indicated concentrations of the *L. lactis* recombinant strains used in our study to immobilised human fibronectin was evaluated. Data are represented as mean ± SEM of 2-3 experiments.

Next, the ability of *S. aureus* MSCRAMMs to confer invasiveness to non-invasive *L. lactis* organisms was tested. Monolayers of ~ 2 x 10^5 endothelial cells were exposed for 1 h to different concentrations of opsonized recombinant *L. lactis* expressing *S. aureus* FnBPA, FnBPB or ClfA, or the control strain *L. lactis* pIL253. Recombinant *lactococci* expressing FnBPA or FnBPB exhibited a very efficient inoculum-dependent pattern of infecting ECs (Fig. 2, Table 1) that was quite similar to our results reported previously for *S. aureus* (23). With less than 10^7 FnBPA-positive *lactococci*, i.e., bacteria to cell ratio of ~50 the maximum of 90 - 100 % infected cells (Fig. 1) with 30 - 50 bacteria per cell (Table 1) was achieved. For FnBPB-positive *L. lactis* recombinants 5 - 10 times higher inocula were needed to reach this maximum infection level (Fig. 2, Table 1). Concentrations of FnBPA- or FnBPB-positive *L. lactis* higher than 10^8, i.e., more than 500 bacteria added per endothelial cell, induced cell detachment and monolayer destruction.

Both ClfA-positive *L. lactis* and the noninvasive control *L. lactis* pIL253 strain (Fig. 2, Table 1) show a very low probability to infect ECs. Maximal infection percentages of 30 - 50 % (Fig. 2) with a few cell-associated bacteria per cell (Table 1) were seen at relatively high inocula increasing 100 bacteria per single EC.

These levels of infection (i.e. determined immediately after bacterial exposure) were not significantly different from those observed after a 23 h-post infection culture period, being the time point at which most of the EC functions were evaluated in the current study, as discussed below (data not shown). Bacterial outgrowth in the medium, used in the infection assay, was negligible during 24 h of bacterial culture at 37^{o}C (data not shown).

Bacterial strain	**Bacteria (CFU) added per monolayer of 2 x 10^5 endothelial cells**				
	10^4	**10^5**	**10^6**	**10^7**	**10^8**
L. lactis pIL253	2.9 ± 1.5[b]	3.5 ± 0.6	6.4 ± 2.1	6.5 ± 2.6	7.6 ± 2.9
L. lactis ClfA (+)	3.3 ± 1.4	8.8 ± 3.6	14.0 ± 4.6	13.5 ± 1.9	11.6 ± 2.5
L. lactis FnBPA (+)	5.6 ± 3.2	18.2 ± 10.0	33.6 ± 14.8	50.0[*]	[**]
L. lactis FnBPB (+)	9.0 ± 9.9	15.7 ± 13.8	18.9 ± 8.6	27.3 ± 8.6	26.9 ± 5.8

TABLE 1: Numbers of lactococci associated with endothelial cells after bacterial challenge. [a]

[a] ECs were incubated for 1 h with the indicated no. of the lactococcal recombinants. After a wash the average no. of cell-associated bacteria per infected endothelial cell was determined.

[b] Values represent mean ± SD calculated from each individual experiment shown in figure 1.

[*], Maximal no. that can be counted reliably; [**], > 50 lactococci per infected cell counted and significant cell injury.

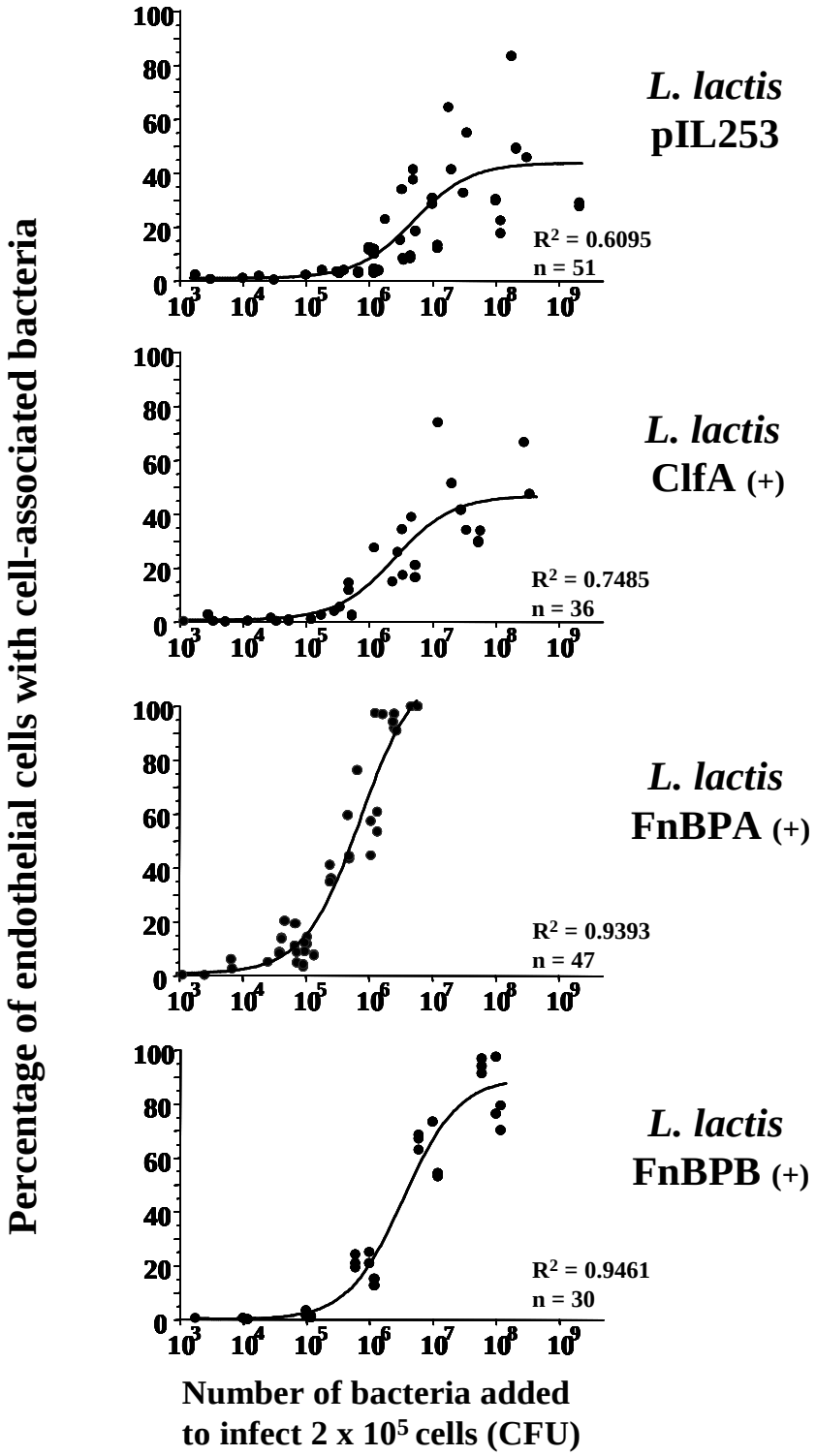

Figure 2: *L. lactis* recombinants associated with ECs.
EC monolayers (2 x 10^5 EC/well) were incubated for 1 h with different inocula of the indicated *lactococcal* mutant strains, washed, fixed, stained with Giemsa and examined by light microscopy. The percentage of cells with associated bacteria, i.e. membrane-bound as well as internalized bacteria, was determined. Each data point represents the value from an individual experiment. ECs of at least 4 different donors were used. The best-fitted curve was assessed by nonlinear regression analyses. From these curves the number of bacteria required to infect 20, 50 or 100 % of the cells was calculated.

Activation of ECs with recombinant lactococci expressing staphylococcal MSCRAMMs

Next we explored whether the infection with the different *lactococcal* recombinant strains elicited EC activation by evaluating surface expression of ICAM-1 and VCAM-1 and production of IL-6, IL-8 and IL-10, as pro- or anti-inflammatory factors related to endovascular infections (1). Uninfected ECs express moderate constitutive levels of ICAM-1 (MFI ± SEM of 203.9 ± 24.9; n = 8) and very low levels of VCAM-1 (MFI ± SEM of 33.2 ± 4.9; n = 8) molecules on their cell surface (these levels were set to 1 in Fig. 3) and secrete about 3.7 ± 1.9 ng/ml IL-8 (n = 6) and negligible amounts of IL-6 per monolayer of 2 x 10^5 ECs (Fig. 4). These values remain unaltered after infection with ClfA-positive *lactococci* or *L. lactis* pIL253 (Fig. 3, 4), despite the fact that interactions between these *lactococci* and ECs at these concentrations do occur, as shown in figure 1. In contrast, endothelial infection with FnBPA- or FnBPB-positive *L. lactis* recombinants was accompanied by a marked inoculum-dependent increase in surface expression of ICAM-1 and VCAM-1 (Fig. 3) and secretion of IL-8 and IL-6 (Fig. 4). Values achieved for FnBPA-positive *lactococci* were higher than those found with similar concentrations of FnBPB-positive *lactococci*. At inocula of ~50 FnBPA-positive *lactococci* per EC surface expression of ICAM-1 and VCAM-1 reached a maximal level that was similar to that obtained with ECs stimulated with 5 µg/ml IL-1, a potent inducer of endothelial pro-inflammatory responses (i.e. 6.4-fold and 8.3-fold increase for ICAM-1 and VCAM-1, respectively after 24 h stimulation). In contrast to the above findings none of the *lactococcal* recombinants induced endothelial production of the anti-inflammatory cytokine IL-10 (data not shown).

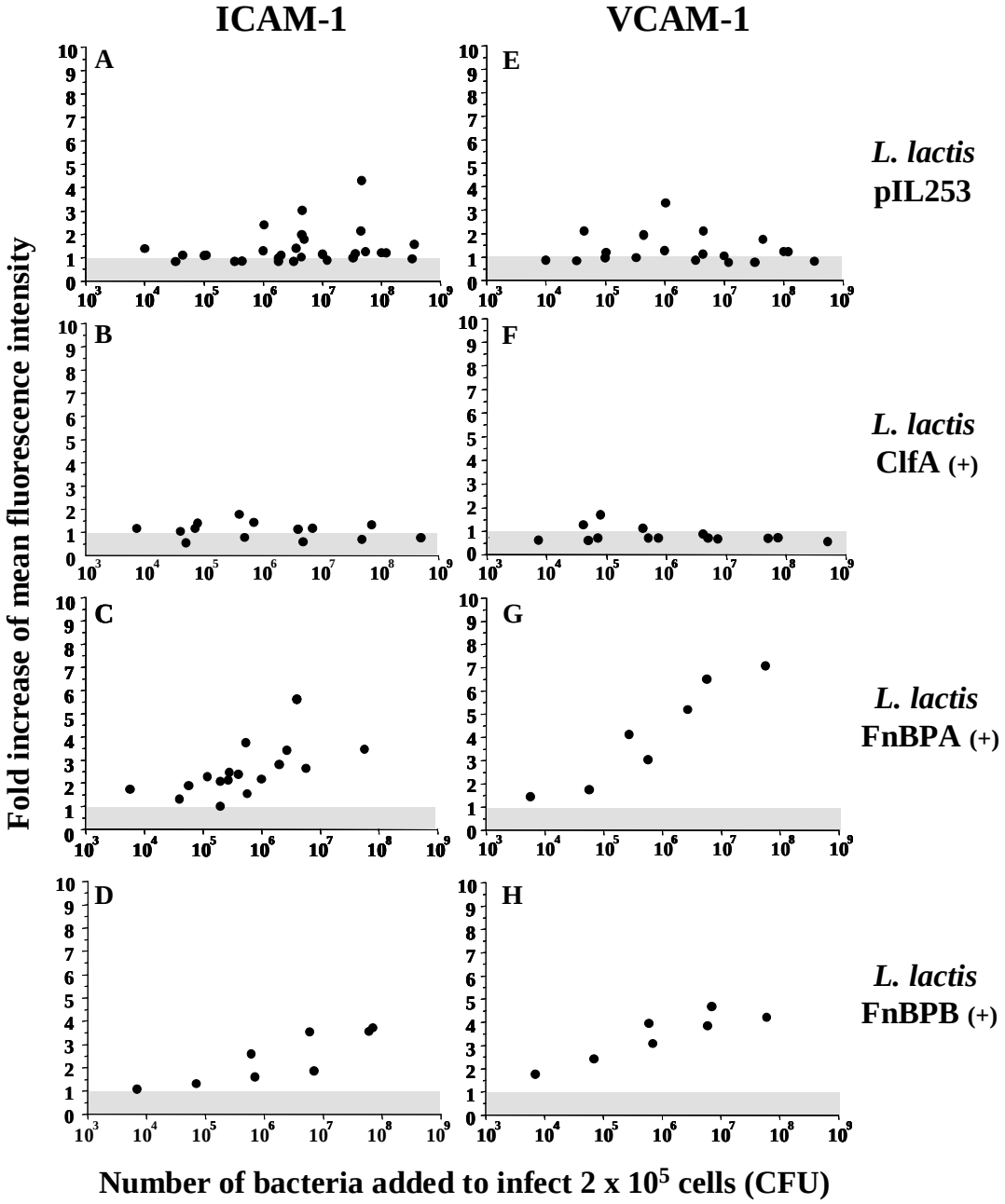

Figure 3: Flow cytometric analysis of EC surface molecule expression after infection with recombinant *L. lactis* strains.

Monolayers of 2 x 10^5 ECs were incubated for 1 h with different concentrations of the indicated *lactococci,* washed, cultured for an additional period of 23 h and prepared for analysis of cell surface expression of ICAM-1 (A-D) and VCAM-1 (E-F) by FACS. Data are shown in fold increase of the mean fluorescence intensity (MFI) of infected ECs relative to the level of ICAM-1 and VCAM-1 expression on control uninfected ECs (MFI values for uninfected cells were set as 1). Each circle represents a relative value of cells from one EC donor.

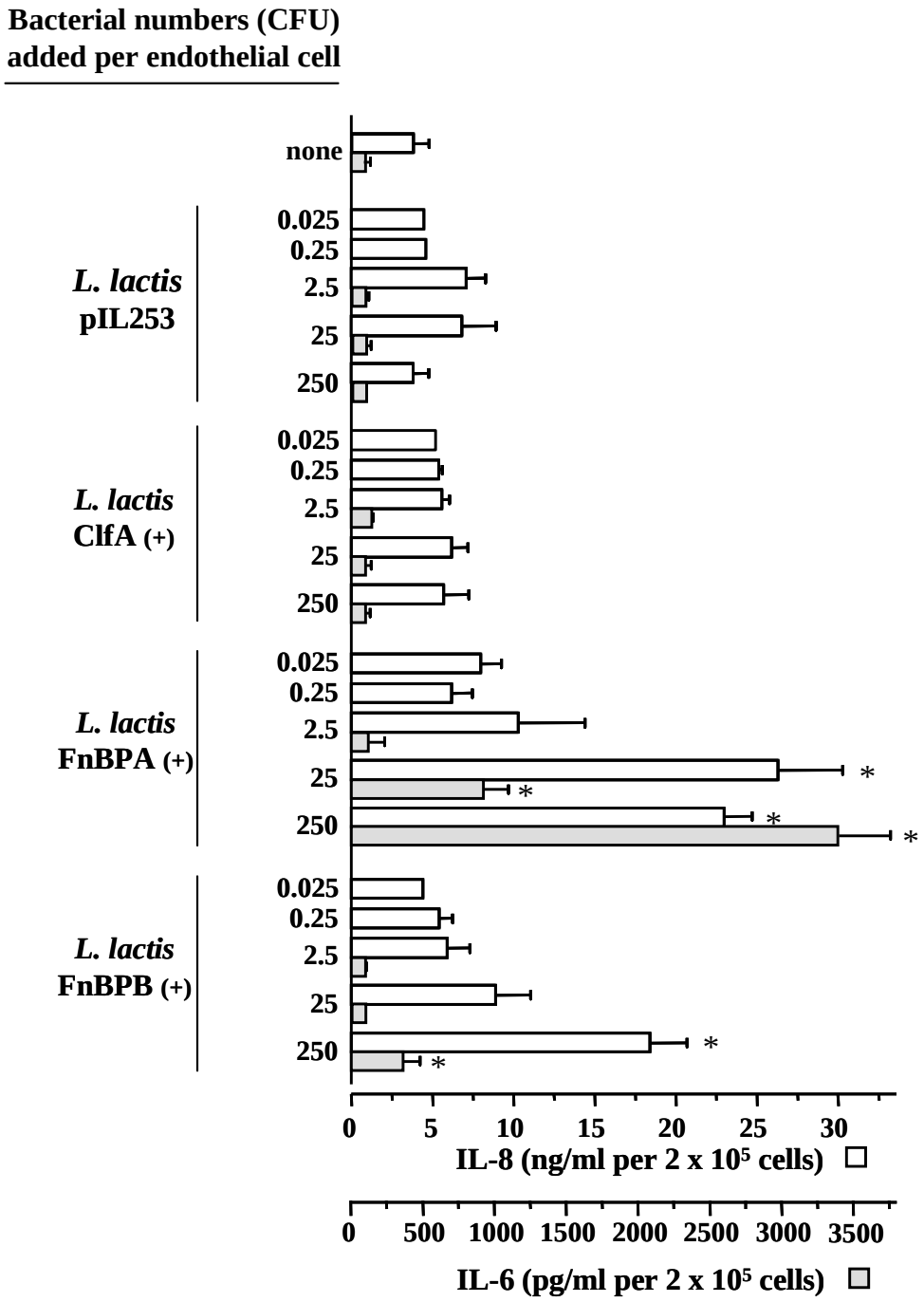

Figure 4: Endothelial IL-8 and IL-6 production after infection with recombinant *L. lactis* strains.

Values of IL-8 and IL-6 protein production were measured from a monolayer of 2 x 10^5 ECs. The amount of cytokine secreted by a monolayer of ECs 23 h after 1 h-infection with the indicated recombinant *lactococci* was determined by ELISA. Data are mean ± SEM of 2 to 6 experiments with ECs from different donors. None represents basal cytokine of uninfected cells. *, $p<0.05$ (paired *t*-test) compared to uninfected cells.

Procoagulant activity of ECs upon infection with FnBPA-positive *L. lactis*

The above experiments demonstrate the importance of in particular FnBPA-mediated endothelial invasion by *lactococcal* recombinants for activation of pro-inflammatory endothelial responses. To further explore the activating potential of this adhesin we measured the endothelial TF-dependent procoagulant response to infections with FnBPA-positive *L. lactis* recombinants. ECs cultured in the presence of *L. lactis* pIL253 or IL-1 served as negative or positive controls, respectively. EC procoagulant activity was investigated on levels of transcription, cell surface expression and biological activity of the TF protein. Uninfected ECs almost did not express TF mRNA. This low basal level of TF transcripts increased gradually with increasing concentrations of infecting FnBPA-positive *L. lactis* (Fig 5A). No relevant endothelial TF mRNA was observed upon infection with *L. lactis* pIL253 (data not shown).

EC surface expression of TF antigen after infection with FnBPA-positive *L. lactis* was assessed by flow cytometry (Fig. 5B). To enable a comparison with the control mutant strain pIL253 the number of infecting bacteria was adjusted to result in levels of 20 %, 50 % or 100 % infected ECs, according the data depicted in figure 1. Confirming our previous findings (22), uninfected ECs did express almost no TF-antigen on their surface (data not shown), and IL-1-stimulated cells displayed maximum elevated levels of TF-antigen (Fig. 5B). Almost a similar increased level, i.e., 1.6- to 1.8-fold increase compared to control uninfected cells, was found in ECs infected with FnBPA-positive lactococci (Fig. 5B). The *L. lactis* pIL253 did not cause any relevant increase in TF expression at an infection level of 20 % and 50 % (Fig. 5B).

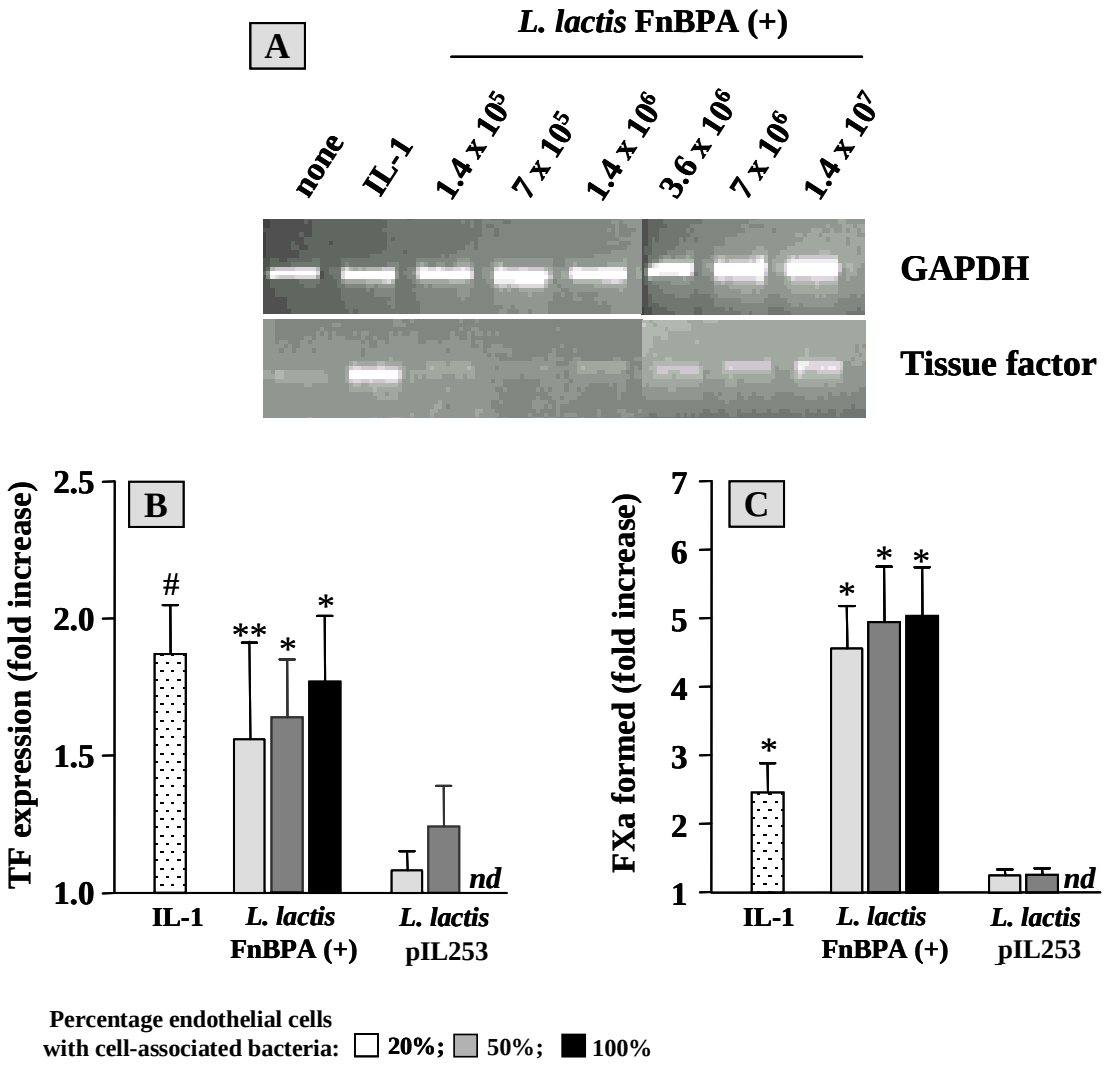

Figure 5: *L. lactis* recombinants expressing staphylococcal FnBPA induce endothelial TF expression and TFA.

(A) The level of TF-mRNA in ECs was assayed by RT-PCR at 4 h after infection of monolayers of 2 x 10^5 ECs with the indicated concentrations of FnBPA-positive *lactococci* or stimulation with 5 ng/ml IL-1. Untreated cells served as controls (none). GAPDH was used as a transcription control. (B) TF surface antigen expression was determined by FACS analysis on ECs after incubated for 24 h with medium alone (untreated cells) or medium containing 5 ng/ml IL-1, FnBPA-positive *L. lactis* or *L. lactis* pIL253. Data are shown in fold increase of infected ECs relative to the level of TF antigen expression on control untreated ECs (MFI values for untreated cells were set as 1). (C) Endothelial TFA was determined 24 h after treatment with IL-1, FnBPA-positive *L. lactis* or *L. lactis* pIL253 by measuring FXa generation.

Data are expressed as fold increase relative to the basal TFA of untreated ECs. To allow comparison between the two mutant strains bacterial concentrations were chosen according the data of figure 2 to result in either 20, 50 and 100 % infected ECs (B, C). Results shown are those of 8 (B) or 10 (C) representative experiments. *, $p<0.05$, **, $p<0.01$, #, $p< 0.02$ (paired *t*-test) compared to untreated cells; nd, not done due to inability to reach a 100 % infection level with *L. lactis* pIL253 (see fig.2)

To determine whether TF surface protein was expressed in its biological active constitution a TF-dependent procoagulant activity (TFA) assay was performed (Fig. 5C). EC monolayers from different donors showed a high degree of variation in their TFA-response to activating stimuli. To circumvent this problem the data are expressed as fold increase in TFA as compared to level in uninfected cells of the same donor. Uninfected ECs expressed very little TFA (7.27 ± 2.27 mU FXa/2 x 10^5 cells, n = 6). A significantly 4.5-fold increase in TFA was found after infection with the FnBPA-positive *lactococci* ($p<0.01$, compared to uninfected ECs, n = 8; paired *t*-test). The three conditions referring to 20 %, 50 % and 100 % infected ECs showed no significant difference in their TFA. As expected, no significant induction of endothelial TFA was seen in the experiments with *L. lactis* pIL253 (Fig. 5C).

TFA of ECs infected with *L. lactis* recombinants during coculture with monocytes

S. aureus infection of ECs results in monocyte recruitment and monocyte-dependent amplification of the endothelial TF-dependent coagulation response as shown by us previously (21, 22, 24, 27). The same in vitro model of experimental endocarditis was used to evaluate the contributing potential of FnBPA molecules in these events. EC monolayers were infected with FnBPA-positive *L. lactis* recombinants or *L. lactis* pIL253 to various degrees of infection and then cocultured with monocytes. The percentage of EC-associated monocytes was determined after 1 h and 6 h of coculture and related to the total level of TFA generated by the cocultured cells at 6 h, as explained in Material and Methods. Infection with FnBPA-positive *lactococci*, but not *L. lactis* pIL253, resulted in an increased percentage of EC-bound monocytes (Fig. 6A). About 78 % of the monocytes adhered within 1 h of coculture to an EC monolayer of which all cells were infected with FnBPA-positive *lactococci* (i.e. 100 % infection level). This percentage is comparable with that obtained with monolayers of IL-1-stimulated ECs (Fig. 6A) and also resembles our previously published value for *S. aureus*-infected ECs (24). At 6 h of coculture the number of monocytes bound to ECs infected with FnBPA-positive *L. lactis* (100 % infection level) had slightly declined to 51 %, but was still higher ($p=0.024$; n = 6; paired *t*- test) than the number bound to uninfected ECs (i.e., 27 % at 6 h of coculture) (Fig. 6B). This ability of

FnBPA-positive *L. lactis*-infected ECs to avidly bind monocytes (Fig. 6A, B) coincided with a significant increase in the amount of FXa generated during subsequent coculture of the cells (Fig. 6D). At each of the 3 different infection levels a maximal level of TFA of 56 to 63 mU FXa/well was induced after 6 h of coculture with monocytes (Fig. 6D), which was about 2.5- to 3-fold higher ($p<0.01$; n = 4, paired *t*-test) than amounts of FXa generated in the absence of monocytes depicted in Fig. 6C. In contrast, the coculture of monocytes with IL-1-activated ECs did not coincide with such TFA enhancement (Fig. 6C vs. 6D).

The increase in monocyte adhesion to FnBPA-infected ECs and consequent induction of TFA during coculture with monocytes were not affected by the presence of mAb against CD18 and CD49d mAbs (Fig. 6B and D, right set of bars). These mAbs did inhibit monocyte adhesion to IL-1-activated ECs by about 70 % ($p<0.05$; n = 4) (Fig. 6B), but this did not coincide with TFA reduction (Fig. 6D).

Interestingly, also the coculture of monocytes with *L. lactis* pIL253-infected ECs, a condition that did not promote EC activation (Fig. 3 and 4) or monocyte-EC interaction (Fig. 6A and B), resulted in a level of TFA that was about 20 mU FXa/well higher than the low basal level of TFA of non-infected ECs after coculture with monocytes ($p=0.04$ or $p=0.008$ at 20 % or 50 % *L. lactis* pIL253-infected ECs, respectively; n = 4, paired *t*-test) (Fig. 6D). Apparently the presence of relatively low numbers of monocytes bound to the surface of *L. lactis* pIL253-infected ECs (20 - 31 %; Fig. 6B) is sufficient to elicit a TFA response in the cocultured cells. The presence of the mAbs did not abolish this induction of TFA (Fig. 6D, right vs. left set of bars).

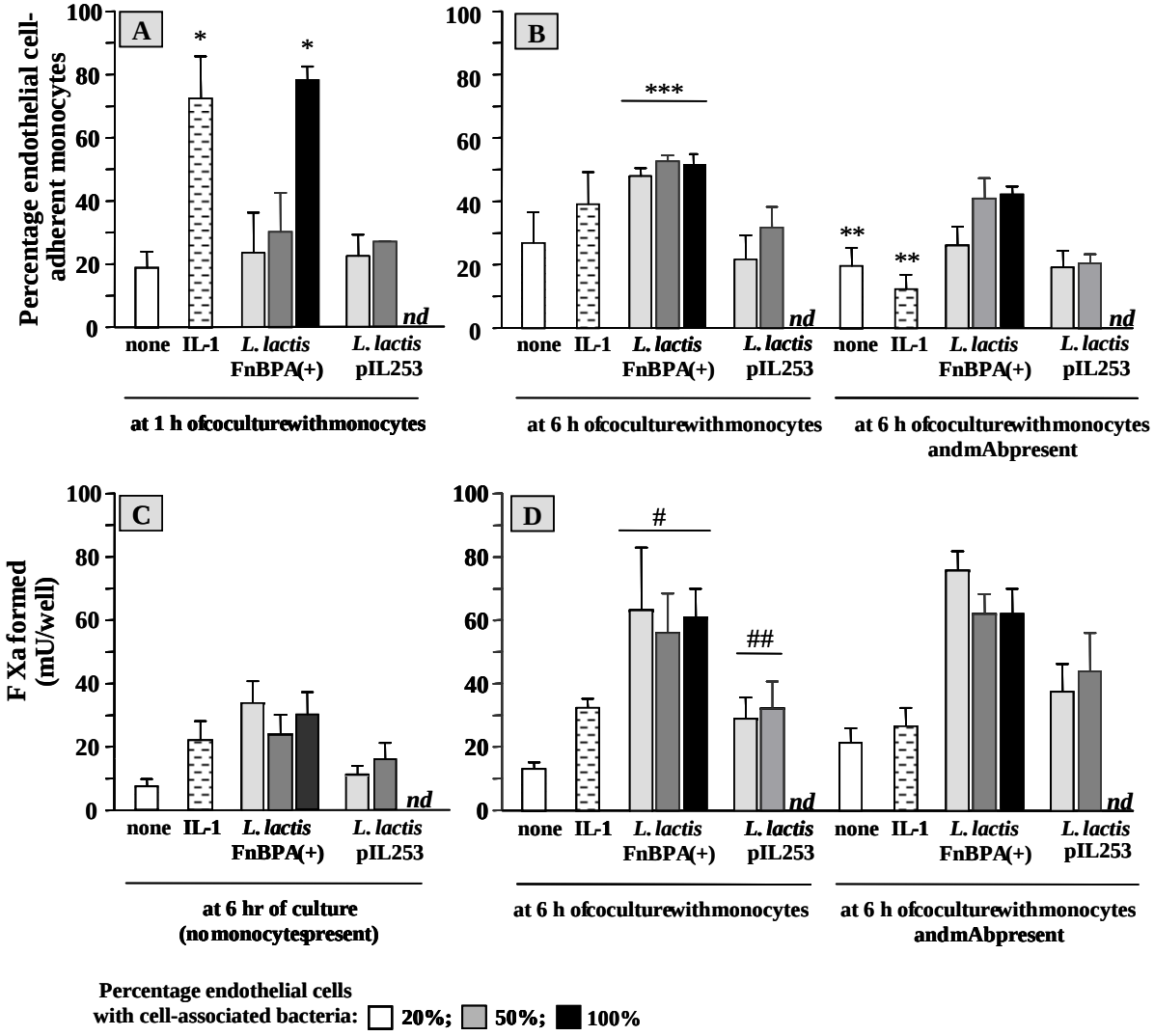

Figure 6: Monocyte-EC interaction and monocyte-dependent augmentation of TFA during coculture of monocytes with ECs infected with *L. lactis* recombinants expressing *staphylococcal* FnBPA.

EC monolayers were incubated for 24 h with medium alone (untreated cells; none) or medium containing 5 ng/ml IL-1, FnBPA-positive *L. lactis* or *L. lactis* pIL253. Bacterial concentrations were chosen according the data of figure 2 to result in 20 %, 50 % and 100 % infected ECs. After washing, monocytes were added (A, B, D) and cocultured with the ECs in the absence or presence of 10 µg/ml of anti-CD49d mAb and 10 µg/ml of anti-CD18 mAb in combination (B, D). At 1 and 6 h during coculture the percentage of EC-associated monocytes was determined (A and B) and the amount of FXa released by the cells in coculture was measured by the TFA-assay (C and D). Results shown are those of 4 (A), 4 (B), 8 (C) and 4 (D) representative experiments. The paired *t*-test was used to evaluate the data. *, p=0.005 compared to none in panel A; **, p<0.05 compared to the respective condition without mAb; ***, p<0.03 compared to monocyte adhesion to non-infected cells at 6 hr of coculture; #, p<0.01 compared to the respective condition without monocytes in fig. 5C; ##, *p*=0.04 (20 % infection) or p=0.008 (50 % infection) compared to non-infected cells after coculture with monocytes; *nd*, not done, because a 100 % infection level cannot be reached with *L. lactis* pIL253 (Fig. 2).

DISCUSSION

In the present study we identify FnBP adhesins from *S. aureus* as critical molecules implicated in eliciting a proinflammatory and procoagulant phenotype of intact human ECs, that typifies an essential initial phase in the early stage of endovascular infections, such as IE (21-24). Using surrogate non-invasive *L. lactis* recombinants that express *staphylococcal* FnBPA, FnBPB or ClfA molecules we demonstrate that the sole interaction of FnBPA, and to a certain extent also that of FnBPB, but not ClfA molecules with ECs is sufficient to trigger diverse EC proinflammatory responses that promote TF-dependent coagulation, cytokine and chemokine production and monocyte adhesion that results in monocyte-mediated amplification of TF-dependent coagulation.

The specific abilities of FnBPA and FnBPB, but not ClfA, adhesins to confer bacterial adhesion to human ECs, as shown in the present study, are in essence confirmatory to earlier observations (4, 6, 9, 11, 13, 17). Further comparative analysis of our data in light of these earlier studies however, reveal at least two additional interesting findings. First, the current values on EC infection obtained with FnBPA-positive *lactococci* resemble those found with invasive *S. aureus* strains as reported by us earlier using the same in vitro model for IE (22, 25), suggesting that no adhesive cofactors are required for conferring optimal bacterial adhesion to ECs. Second, a consistently ~ 10-fold lower degree of infection was observed with FnBPB-positive *L. lactis* as compared to the FnBPA-positive recombinants. As both FnBPs have similar C-terminal fibronectin-binding regions (i.e., repeat regions D1-D4 and Du) (30) and were shown to exhibit a similar capacity to bind immobilised human fibronectin (this study), it is tempting to suggest that this observation could be explained by the less similar amino acid sequences of the N-terminal A regions (~ 40 % homology) or the two additional repeat regions B1 and B2 of FnBPA (32). The credibility of this explanation, however, awaits further investigation.

The novelty and major focus of our present study, however, was to critically evaluate the potential of FnBPA, FnBPB and ClfA to induce significant EC activation upon cellular contact in the absence or presence of adhering monocytes. Hence, a system was chosen in which only one staphylococcal adhesin was expressed at the surface of the surrogate *lactococci*. In keeping with results obtained with resting platelets

(33), we show that activation of ECs by contact with bacteria requires surface expression of staphylococcal FnBPA or FnBPB. As a result these ECs acquire a pro-inflammatory and procoagulant phenotype, as shown by enhanced cell surface expressions of ICAM-1 and VCAM-1, secretions of IL-6 and IL-8, but not IL-10, as well as monocyte adhesion and, as discussed in more detail below, an enhanced TF-dependent procoagulant activity.

Despite their differences in conferring EC infection, FnBPA- and FnBPB-expressing lactococci were equally effective in inducing EC activation. This can be concluded by evaluating activation levels of ECs cultures that were equally infected with either strain of bacteria. EC activation was however, not observed upon infection with ClfA-expressing lactococci also not when relatively high inocula were used to achieve a level of 50 % infected cells. Apparently the contact between ClfA-positive *lactococci* and the surface of intact ECs is insufficient to signal EC activation. Previously it was shown that such contact also did not result in endothelial internalization of the ClfA-expressing *lactococci* (5, 19). The current discrepant results with *lactococci* expressing FnBPs or ClfA may, in essence, relate to those of a recent study with experimental endocarditis catheterized rats that were challenged with FnBPA- or ClfA-positive *lactococci* (20). Expression of ClfA was shown to be essential for bacterial colonization of mechanically damaged valves in particular by binding to the fibrinogen-rich lesions instead of intact neighbouring ECs. EC contact was explicitly observed with FnBPA-positive *lactococci* only (20). Together these studies may support the concept that in the early phase of staphylococcal valve colonization, when the valvular surface is still undamaged and lacks fibrin depositions, FnBPA, but not ClfA, is of dominant importance for binding and concomitant activation of ECs. In the later phase of valvular infection, when fibrin-rich lesions are formed, FnBPA and ClfA may play complementary roles.

With regard to the mechanism by which FnBP-expressing *L. lactis* recombinants activate ECs our findings, in light of general features of *staphylococcal* FnBPs and the above mentioned study in catheterized rats (20), may highlight an explicit intermediate contribution of plasma fibronectin and/or fibrinogen. Indeed, a preliminary study that we performed with recombinant *lactococci* expressing truncated FnBPA molecules, as described by Massey et al. (9), reveal that FnBPA requires its fibronectin-binding structural domains C and D alone or in combination

with domain B to preserve its potential to trigger EC activation This issue and the possible EC receptors are currently under investigation.

From our further investigations we conclude that a bacteria-EC interaction mediated by FnBPA is a decisive determinant in the amplification of procoagulant activity in a setting in which monocytes are present. The importance of circulating monocytes settling on a *S. aureus*-infected endothelial or valvular surface for the maintenance of TFA and formation of fibrin clots has been assigned by us previously (21, 24, 27). Adding to this, the current data reveal that the enhanced adhesion of monocytes to ECs infected with FnBPA-positive *L. lactis* recombinants results in a synergistic enhancement of TF-dependent coagulation. No influence of antibodies that block CD18 and CD49d mediated monocyte adhesion was found, suggesting that in these coculture experiments cellular adhesion as well as the generation of high levels of TFA is not explained by an interaction between integrin receptors on monocytes with their respective counterparts ICAM-1 and VCAM-1 on the infected ECs, but most likely involves other adhesive molecules presented by monocytes, infected ECs and/or their membrane-bound bacteria.

Upregulation of TFA in cocultures of monocytes and bacteria-infected ECs was also observed to some degree with the control strain *L. lactis* pIL253 as the infecting organism, and apparently this response is not restricted to FnBPA-positive *lactococci*. This finding was rather unexpected, but is in accordance with a previous coculture study of monocytes with ECs after infection with *Streptococcus sanguis* or *Staphylococcus epidermidis* (22). These bacterial species are not associated with severe EC infection and acute endocarditis and in vitro have low abilities to infect ECs (22). Coculture studies with these bacteria revealed a synergistic relationship between ECs, bacteria and monocytes to induce TFA. Monocytes recruited on the surface of bacteria-infected ECs amplified TFA during coculture of the cells. The mechanism by which monocytes increased TFA in these cocultures required bacterial presence (possibly phagocytosis) and was less dependent on the type of the infecting bacterium or the virulence factors utilized to establish the endothelial infection.

Collectively, our findings give further insight in the essential bacterial and cellular interactions in the early phases of the pathogenesis of *S. aureus* endocarditis when the valvular endothelium is still undamaged and fibrin depositions have not been

formed yet. The data confirm that *S. aureus* FnBP molecules are sufficient to lead to endothelial adhesion and invasion and in a second step are responsible for the activation of proinflammatory and procoagulant EC responses. We hypothesize that the subsequent recruitment of monocytes to these sites that occurs within hours after the onset of infection guaranties the formation and further enlargement of the infected vegetation. Further studies should address whether the domains of the FnBPA molecule that are responsible for conferring bacterial adherence are similar to those triggering EC activation. This knowledge would provide potential targets for therapeutic intervention.

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CHAPTER IV

Contribution of (sub)domains of *staphylococcal aureus* fibronectin-binding protein to the proinflammatory and procoagulant response of human vascular endothelial cells

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Thromb Haemost. 101(3): 495-504; 2009

ABSTRACT

The *Staphylococcus aureus* Fibronectin (Fn) binding Protein A (FnBPA) is involved in bacterium - endothelium interactions which is one of the crucial events leading to infective endocarditis (IE). We previously showed that the sole expression of *S. aureus* FnBPA was sufficient to confer to non-invasive *Lactococcus lactis* bacteria the capacity to invade human endothelial cells (ECs) and to launch the typical endothelial proinflammatory and procoagulant responses that characterize IE. In the present study we further questioned whether these bacterial - EC interactions could be reproduced by single or combined FnBPA subdomains (A, B, C or D) using a large library of truncated FnBPA constructs expressed in *L. lactis*. Significant invasion of cultured ECs was found for *L. lactis* expressing the FnBPA subdomains CD (aa 604-877) or $A4^{+16}$ (aa 432-559). Moreover, this correlates with the capacity of these fragments to elicit in vitro a marked increase in EC surface expression of both ICAM-1 and VCAM-1 and secretion of the CXCL8 chemokine and finally to induce a tissue factor-dependent endothelial coagulation response. We thus conclude that (sub)domains of the staphylococcal FnBPA molecule that express Fn-binding modules, alone or in combination, are sufficient to evoke an endothelial proinflammatory as well as a procoagulant response and thus account for IE severity.

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen commonly associated with a wide range of acute and chronic infections, including skin and soft tissue infections, bacteremia, osteomyelitis and infective endocarditis (IE). IE results from direct interactions between circulating bacteria and host endothelia and can affect patients with or without previously damaged heart valves (1, 2). Recent work showed that IE is a multistep event involving bacterial surface-bound adhesive molecules, entitled microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), that interact with various host's extracellular matrix proteins, such as fibronectin (Fn), fibrinogen (Fg) or elastin, to bind to, and establish active internalization, and colonization of host cells (3-5).

In vitro studies of recent years, including our own, collectively revealed that Fn-mediated interactions between the Fn-binding *S. aureus* MSCRAMM proteins (FnBPs) FnBPA and FnBPB, and Fn receptors on endothelial cells (ECs) are pivotal in promoting endothelial colonization, invasion and activation, thus in promoting IE (6-13). We showed in particular that Fn-binding protein A (FnBPA)-mediated bacterial adhesion to human endothelium is essential and sufficient to trigger diverse endothelial proinflammatory and procoagulant responses (12, 14-21). For example, FnBPA induces the production of IL-6, CXCL8 and CCL2 and the expression of various cellular adhesion molecules, such as ICAM-1 (CD54) and VCAM-1 (CD106), both responsible for monocyte adhesion (12, 15). FnBPA induces T-cell activation as well as platelet aggregation (21-25). Finally, FnBPA is also responsible for a tissue factor (TF)-mediated endothelial procoagulant response that synergistically acts with adhering blood monocytes to trigger the coagulation cascade leading to the formation of a typical thrombus called vegetation (2, 12, 20, 26). In addition to these in vitro findings, staphylococcal FnBPA was shown essential for endovascular disease progression *in vivo*. Using a rat model of experimental endocarditis and a model of heterologous expression of staphylococcal adhesins in *Lactococcus lactis*, Que et al. demonstrated that the expression of FnBPA was a prerequisite for *S. aureus* to invade ECs and to establish a persistent and recurrent endovascular disease (27). In summary, it appears that staphylococcal FnBPs are the major players responsible for

both the pro-inflammatory and pro-coagulant endothelium responses observed during *S. aureus* endovascular infections.

These functional capacities of FnBPA and the described multi-domain molecular structure of this adhesion have directed our interest to specify the contributing role(s) of single FnBPA domains to EC activation and IE pathogenesis. The structural organization of *S. aureus* FnBPA is outlined in Table 1. The protein consists of 1018 amino acids divided into 4 general domains, termed A, B, C and D, flanked by a short N-terminal signal sequence and C-terminal sequences required for cell wall anchoring (reviewed in detail by ref. (6, 11, 28). The B domain has two amino acid repeats B1 and B2, and is linked to the C domain that encompasses region Du. The D region consists of three consecutive repeats, termed D1, D2 and D3 that resemble Du, and one incomplete repeat (D4). The N-terminal A domain is responsible for binding Fg (9) (29, 30) and elastin (30, 31), whereas the C- terminal region with domains B-DuC-D as well as the region encompassing the hinge between domain A and B (e.g. residues 432 to 559) bind Fn (9, 32-34). This traditional organization was recently refined into further subdomains, which might cooperate with each other for binding (Table 1) (32, 35). Residues at the N-terminal of the protein are predicted to fold into three subdomains (N1, N2, N3), with N2 and N3 being involved in fibrinogen and elastin binding (30). In this revised organization, the C-terminal Fn-binding domains are organized in eleven tandem repeats, each interacting with so-called type 1 modules of Fn through a tandem beta-zipper mechanism (32, 35).

The exact contribution of single FnBPA subdomains to the generation of the typical endothelial proinflammatory and procoagulant activities observed during *S. aureus* endovascular infection is unknown. In line with our previous study, and using the same in vitro model of EC activation (12), we further questioned whether different subdomains of the staphylococcal FnBPA could also mimic the typical EC activation responses we observed with the full length FnBPA protein. Using a large library of *L. lactis* expressing single different FnBPA (sub)domains or combinations thereof, we demonstrated that domains with Fn-binding activities, in contrast to others, were necessary and sufficient to provoke the typical EC proinflammatory and procoagulant responses observed during the early phase of *S. aureus* endovascular infections.

MATERIAL AND METHODS

Reagents

Culture medium 199 (M199), RPMI 1640 and fetal calf serum (FCS) were from GIBCO Laboratories (Grand Island, NY), penicillin from Brocades Pharma B.V. (Leiderdorp, The Netherlands), streptomycin from Gist-brocades N. V. (Delft, The Netherlands) and amphothericin-B from Squibb B.V. (Rijkswijk, The Netherlands). Collagenase type 1A, L-glutamine, bovine serum albumin (BSA), purified human Fn and crystal violet were all purchased from Sigma-Aldrich Inc. (St. Louis, Mo.), gelatin and trypsin from Difco Laboratories (Detroit, MI) glutaraldehyde from Polyscience Inc. (Warrington, UK) and EDTA from Boerhinger (Mannheim, Germany). Human serum was collected from healthy donors and inactivated at 56°C for 30 minutes (HuSi). Endothelial cell growth factor (ECGF) was made from bovine hypothalamus as described previously (36). Clotting factor X (FX) and the chromogenic substrate PefachromeFXa® were obtained from Kordia (Leiden, The Netherlands). Factor VII (FVII) was prepared from human plasma as described (17). Acetic acid, $CaCl_2$ and TRIS-base came from Merck (Darmstadt, Germany).

Bacterial strains

Bacteria used in this study are recombinant strains of the non-virulent *L. lactis* subsp. *cremoris 1363* expressing (sub)domains of the staphylococcal MSCRAMM FnBPA on their surface. The strains were constructed by gene transfer of the truncated *fnbA* gene of *S. aureus 8325-4*, using an inverse PCR procedure as described (9, 37, 38) and selected oligonucleotide primers sets to amplify and combine domains and subdomains of FnBPA, as described in detail elsewhere (39). This reference also enumerates the phenotype and functional characteristics of the strains. In addition we used the *L. lactis* strains A1, A12 and A123, that were constructed by the use of the forward primer F7 *Pvu*I in combination with the reverse primers R2 *Pvu*I, R3 *Pvu*I or 5'-TACGATCGATTCCCACTCATATTACTTGTGAC-3' (position 1393 - 1416, *Pvu*I restriction sensitive), respectively. The latter reverse primer was used in combination with forward primer F4 *Pvu*I to construct *L. lactis* A123B^{-16}CD. The reverse primer R1 *Pvu*I and forward primer 5'-TACGATCGGAAGAGGATATGATTCATC-3' (position

1747 - 1766, *Pvu*I restriction sensitive) were used to construct *L. lactis* BCD. The sequences of the above mentioned primers are described in detail elsewhere (39).

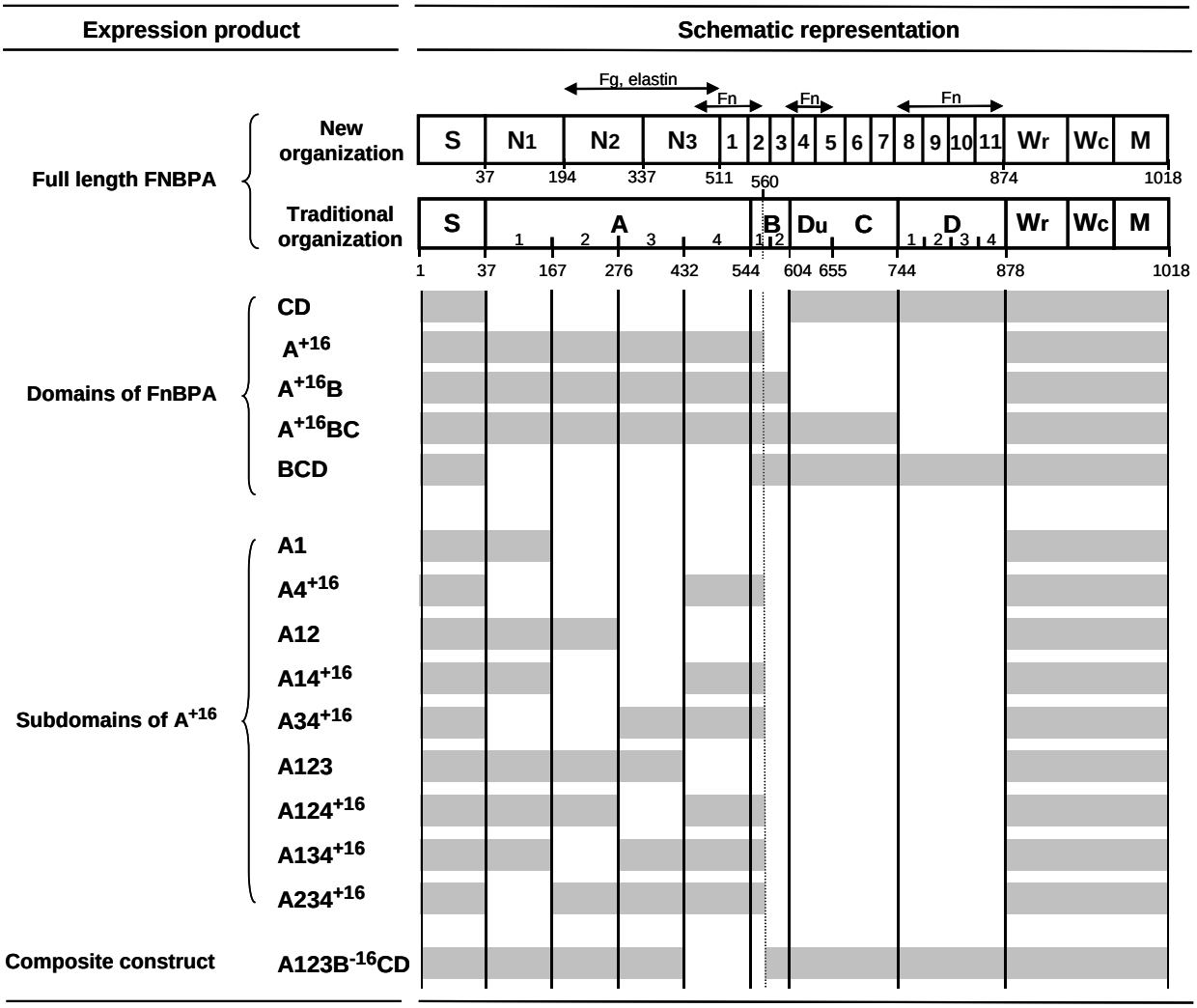

Table 1: Schematic representation of the FnBPA constructs expressed in *L. lactis* used in this study.

The traditional structural organisation of *S. aureus* FnBPA molecule encompassing the A-B-DuC-D domains and the recently refined new organisation of the molecule with subdomains N1, N2 and N3 and 11 Fn-binding tandem repeats are shown from its N- to C-terminal end on top. Sequences that bind fibrinogen (Fg), elastin or fibronectin (Fn) are indicated (for references see Introduction). Each construct has the N-terminal amino acids 1-37 (Signal sequence; S) and C-terminal amino acids 878-1018, enclosing the cell wall-spanning (Wr) and cel wall-anchoring (Wc) segments and the hydrophobic cell membrane spanning segment (M) in common**.**

Table 1 shows a diagrammatic presentation of the different recombinant strains used in this study. Each truncated or composite construct was called according to the different domains or subdomains of FnBPA expressed. All recombinant lactococci

were resistant to erythromycin to allow selection. *L. lactis* containing the lactococcal plasmid pIL253 expressing only the erythromycin resistance determinant was used as the control mutant strain. All bacterial strains were prepared and kindly provided by Dr. P. Moreillon and were stored at -70 °C in liquid M17 medium, i.e. M17 broth (Oxoid, Basingstoke, UK) plus 0.5 % glucose (Brunschwig Chemie, Amsterdam, The Netherlands) and 5 µg/ml erythromycin (Abbott B.V., Hoofddorp, The Netherlands), supplemented with 10 % (vol/vol) glycerol. Before use in the infection assays bacteria were grown overnight at 30 °C without shaking in M17 medium or on M17 agar plates supplemented with 0.5 % glucose and 5 µg/ml erythromycin.

Fn binding assay

The in vitro adherence of the recombinant bacterial strains to immobilized purified human Fn was verified by the Fn binding assay (9). Briefly, 96 well plate were coated for 2 h at 37 °C with a fixed amount of 10 µg/ml Fn and then incubated for 1 h at 37 °C with 2 % BSA to block unspecific sites. Different concentrations of unopsonized bacteria between 5 x 10^5 and 2 x 10^6 bacteria were added for 2 h to the plate (200 mm^2), fixed with 2 % glutaraldehyde and coloured with 0.5 % (w/w) crystal violet. Fn binding was evaluated after conversion of the optical density at 570 nm (Victor^2 multilabel counter, PerkinElmer, Turku, Finland).

Preparation and culture of human ECs

ECs were cultured from human umbilical veins from healthy donors and seeded on gelatin-coated tissue culture dishes and cultured in culture medium consisting of M199, 100 U/ml penicillin G, 0.1 mg/ml streptomycin, 100 U/ml amphothericin-B, 0.1 mg/ml ECGF, 5 U/ml heparin, 1mM L-glutamine, 5 % HuSi and 5 % heat inactivated FCS (FCSi) in a 5 % CO_2 incubator at 37 °C as described in detail elsewhere (40). Confluent monolayers of ECs (second passage) cultured on gelatin-coated 24-well tissue culture plates or on gelatin-coated glass coverslips were used for the experiments yielding a cell density of approximately 800 cells/mm^2.

Infection of ECs with bacteria

Confluent monolayers of ECs were incubated for different periods of time with various inocula of opsonized *L. lactis* bacteria at 37 °C in a 5 % CO_2 incubator in

culture medium including antibiotics, essentially as described previously (12). These conditions are not optimal for *L. lactis* growth and bacterial outgrowth during the 24 h incubation at 37 ^{o}C was negligible, as was verified in control experiments. The number of bacteria used in the infection assay was confirmed by colony counts after plating serial dilutions on M17 agar plates and overnight incubation at 30 °C. Results are expressed as the percentage of infected ECs, i.e. cells with at least one cell-associated bacterium, which was determined under a light microscope using a standardized counting procedure, essentially as described for infection with *S. aureus* elsewhere (41).

Flow cytometric analysis of EC proinflammatory and procoagulant surface molecules

EC monolayers were infected for 24 h with variable numbers of opsonized recombinant *L. lactis* bacteria. The cells were harvested using trypsin and collected and washed in cold PBS supplemented with 0.1% FCSi (wash buffer). Cells were subsequently taken under three incubation steps on ice: 15 min with PBS containing 1% goat serum, then 30 min with 1µg/ml of the appropriate mAb and finally 30 min with phycoerythrin-conjugated goat-anti-mouse Ig (Southern Biotechnology Associates Inc. Birmingham, Ala.). In between each step cells were washed twice with cold wash buffer. At least 5000 cells were analyzed by flow cytometry using a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, N.J.). Background fluorescence was set using (infected) cells incubated with the conjugated mAb alone. The following mouse mAb against human ECs surface molecules were used: mAb HTF-1 against tissue factor (TF; CD142) from Becton Dickinson; mAb 15.2 against ICAM-1 (CD54) from Santa Cruz Biotechnology (Santa Cruz, Ca.) and mAb 1G11B1 against VCAM-1 (CD106) from Biosource International (Camarillo, Ca.).

Analysis of CXCL8 production

Supernatants of EC cultures, harvested after infection with recombinant *L. lactis* bacteria, were assayed for production of chemokine CXCL8 (IL-8) by using the PeliKine Compact^{TM} human IL-8 enzyme-linked immunosorbent assay kit (Sanquin, Amsterdam, The Netherlands) according to the supplier's instructions. The sensitivity of the kit allowed detection of CXCL8 concentrations above 8.0 pg/ml.

Analysis of TF-dependent procoagulant activity (TFA)

TFA of (infected) EC cultures was measured by transformation of clotting factor X (FX) to FXa using Pefachrome FXa® (Kordia, Leiden, The Netherlands) exactly as described earlier (12, 17). FXa concentrations were calculated based on a calibration curve of purified FX that was fully activated with Rusel Viper Venom (Chromogenix, Mölndal, Sweden). Values are given as mU FXa / well containing a confluent monolayer of about 2 x 10^5 ECs.

Statistical analysis

Data were analyzed with the SPSS program for Windows software, version 14 (SPSS GmbH Software, Muenchen, Germany). The paired Student's *t* test (two-tailed) was used to control for variation in measurements due to the fact that for each experiment ECs isolated from different donors were used. Results are expressed by the mean value ± SEM. The level of significance was set at a *P* value of ≤ 0.05.

RESULTS

FnBPA domains responsible for adherence to immobilized human Fn

Since a thorough expression analysis of the staphylococcal FnBPA-constructs expressed by *L. lactis* has recently been performed (39), in this study we only verified their Fn-binding phenotype (Table 2). Recombinant *L. lactis* expressing full length *S. aureus* FnBPA (*L. lactis* FnBPA) was used as a positive control (activity was set to 100%) and *L. lactis* pIL253, carrying an empty plasmid, served as a negative control. As expected, *L. lactis* recombinants expressing truncated FnBPA fragments encompassing one or more Fn-binding modules (Table 1; (11, 32)) were able to adhere to immobilized Fn (Table 2).

FnBPA domains responsible for *L. lactis* adherence to and invasion of human ECs

The ability to adhere and subsequently infect monolayers of human venous ECs was determined for each of the recombinant *L. lactis* strains using the 24 h infection assay previously described (12) and bacterial inocula that ranging from 10^4 to 10^8

CFU per 2 x 10^5 ECs (Fig. 1). Bacterial outgrowth during the 24 h incubation of the assays at 37 ^{o}C was negligible (data not shown). Incubations with bacterial numbers exceeding 10^8 were omitted because of substantial EC detachment and destruction of the monolayer integrity. For each recombinant strain the probability of EC infection was inoculum dependent.

L. lactis mutant strain	% bacteria bound to immobilized Fn
L. lactis FnBPA	100
L. lactis CD	100
L. lactis A^{+16}	52.6
L. lactis $A^{+16}B$	52.6
L. lactis $A^{+16}BC$	73.7
L. lactis BCD	31.0
L. lactis $A4^{+16}$	36.8
L. lactis pIL253	10.5

TABLE 2: Binding of *L. lactis* recombinants expressing FnBPA to immobilized Fn[a]

[a] Unopsonized bacteria were allowed to adhere for 2 h to purified human Fn immobilized into microtitre culture plates. The number of bound bacteria was determined as described in the Materials and Methods and expressed as the percentage relative to the Fn binding capacity of *L. lactis* expressing full length FnBPA (*L. lactis* FnBPA) which was set to 100%. Values represent the average from at least 3 experiments performed on different days and with different concentrations of bacteria.

In line with our recent study (12) and confirming data from our previous study with intact *S. aureus* (18), *L. lactis* expressing full length staphylococcal FnBPA showed a high probability to adhere to ECs, with a maximum of 90-100% infected cells achieved with a very low inoculum of 10^6 bacteria / well (i.e., ~5 bacteria per single EC)(Fig. 1A). A similar or slightly better ability to infect ECs was observed with lactococci expressing the CD domains of FnBPA (*L. lactis* CD)(Fig. 1B).

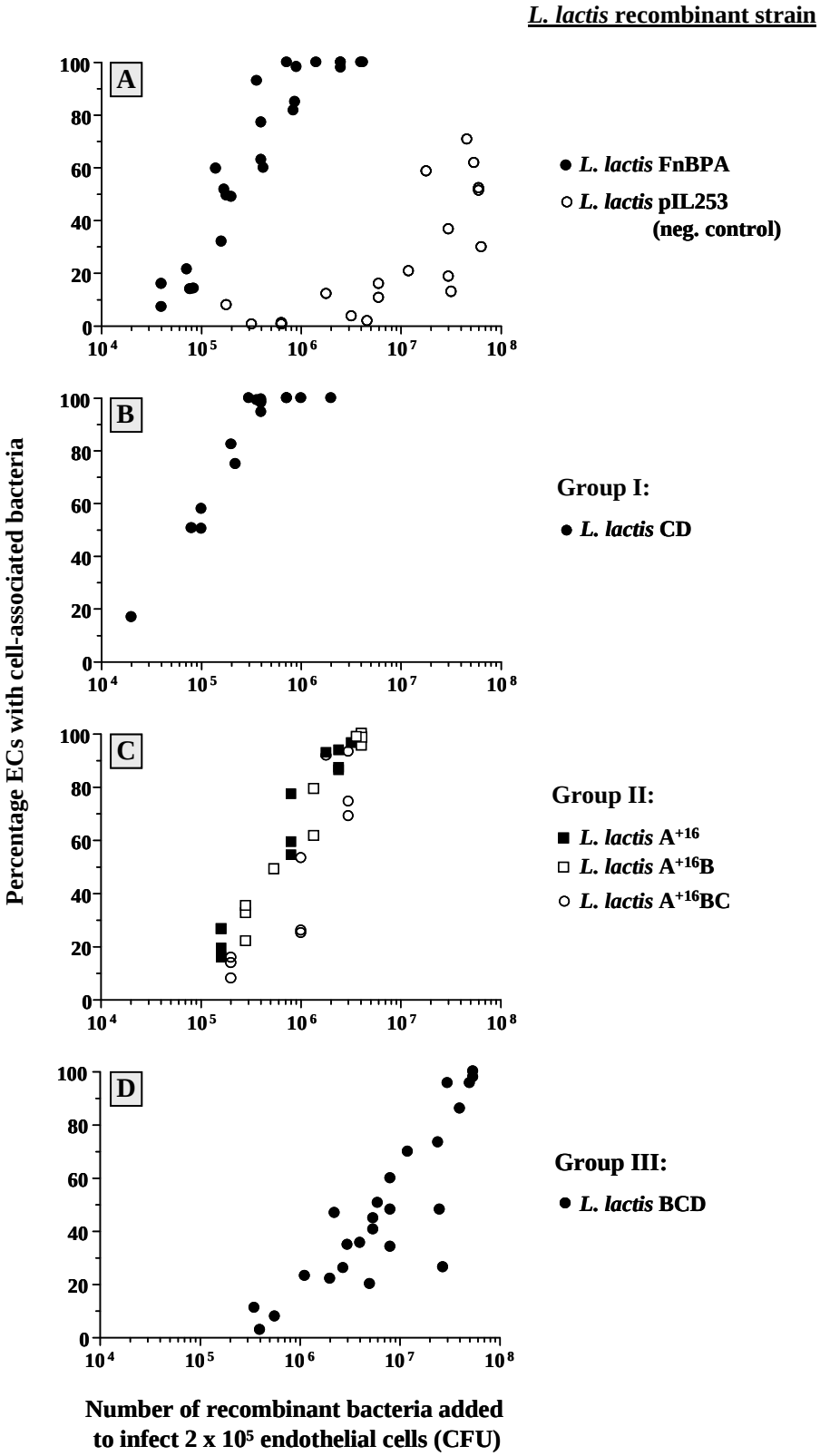

Figure 1: Association of L. lactis recombinant strains with ECs.
EC monolayers (~ 2 x 10^5 EC per well) were incubated for 24 h with different inocula of the indicated lactococcal mutant strains, washed, fixed, stained with Giemsa and examined by light microscopy. The percentage of cells with associated bacteria, i.e., membrane-bound as well as internalized

bacteria, was determined. Each data point represents the value from an individual experiment. ECs of at least 6 different donors were used. On the basis of their ability to infect ECs the recombinant strains were split into three groups (Group I, II, or III). *L. lactis* pIL253 was used as the FnBPA-negative control mutant strain. The number of bacteria required to infect 20, 50 or 100% of the cells was calculated from these graphs and is called the ID20, -50, or -100.

On the other hand, significantly lower levels of infectivity were observed with the recombinant strains expressing the A domain, alone or in combination with the B and/or C domains (*L. lactis* A^{+16}, AB or ABC), or with *L. lactis* BCD. For these strains about 5 to 50 times higher inocula, as compared to *L. lactis* FnBPA or *L. lactis* CD, were needed to achieve 100% of infection (Fig. 1C). With the negative control *L. lactis* pIL253 an infection level of maximum 40 - 50% infected EC was reached only with very high inocula of 150 - 350 bacteria per single EC (Fig. 1A).

On the basis of the above results (Fig. 1) we further split the *L. lactis* mutant strains into 3 groups: (1) Group I consisting of strain *L. lactis* CD showing a high potential similar to *L. lactis* FnBPA, to bind to and infect ECs;. (2) Group II including strains with a moderate infection potential, i.e., *L. lactis* A^{+16}, AB, or ABC; (3) Group III consisting of *L. lactis* BCD being bacteria less potent in binding and infecting ECs than those of group II, but, in contrast to the control *L. lactis* pIL253, capable of infecting 100% ECs at high inocula. This classification will be used throughout this study to facilitate a comparison of the activating abilities of the strains.

Functional responses of ECs to infection with *L. lactis* mutant strains expressing specific domains of staphylococcal FnBPA

Human ECs respond to infection with *S. aureus* or *L. lactis* expressing *S. aureus* FnBPA by activating inflammation and coagulation (12, 14, 19). In the current series of experiments we evaluate the capacities of the various *L. lactis* recombinant strains to activate both a proinflammatory and a procoagulant EC response. For this purpose characteristic endothelial markers of inflammation, such as surface expression of ICAM-1 and VCAM-1 and secretion of CXCL8, were measured in parallel to the evaluation of an endothelial TF-dependent procoagulant activity. Since EC activation was shown to be dependent to the magnitude of bacterial infection (12, 19), inocula responsible for 20%, (infecting dose 20 or ID20), 50% (ID50) or 100% (ID100) of EC infection, calculated from the data depicted in figure 1 were used for group

comparisons. It should be noted that for the control strain *L. lactis* pIL253 the ID100 cannot be reached.

Proinflammatory responses of infected ECs

Figure 2 depicts results from flow cytometric analyses of ECs exposed for 24 h to strains of group I, II and III at ID100. Uninfected ECs express moderate constitutive levels of ICAM-1 (MFI ± SEM of 203.9 ± 24.9; n = 8) and very low levels of VCAM-1 (MFI ± SEM of 33.2 ± 4.9; n = 8) molecules on their cell surface (Fig. 2, none) and per 2 x 10^5 cells in a monolayer secrete about 2.3 ± 0.5 ng/ml CXCL8 (n = 8) (Fig. 3, value set to 1.0). Both *L. lactis* FnBPA and group I strain *L. lactis* CD, elicited a marked increase in EC surface expression of ICAM-1 and VCAM-1 (Fig. 2). Mean fold increases in ICAM-1 and VCAM-1 surface expression calculated from 6 - 14 independent experiments were 7.1 ± 0.9 and 32.3 ± 4.5 for *L. lactis* FnBPA-infected ECs and 8.4 ± 0.72 and 27.9 ± 6.9 for *L. lactis* CD-infected ECs, respectively.

These values were not different from those obtained with ECs infected at similar conditions (ID100) with group II strains *L. lactis* A^{+16}, AB, or ABC. Mean fold increases in ICAM-1 and VCAM-1 surface expression on ECs infected with these strains ranged from 7.6- to 8.2-fold and 20.6- to 27.9-fold, respectively. Interestingly, slightly lower mean values were achieved for *L. lactis* BCD (group III) at ID100, namely a 3.4-fold increase for ICAM-1 and an 8.2-fold increase for VCAM-1 cell surface expression (Fig 2). ECs infected with *L. lactis* pIL253 at the maximal achievable level (ID50) had unaltered basal expression levels of ICAM-1 and VCAM-1 (data not shown).

Endothelial production of CXCL8 was markedly induced by *L. lactis* FnBPA and not by *L. lactis* pIL253 (Fig. 3). Interestingly, an even higher quantity of this proinflammatory chemokine was produced after infection with *L. lactis* CD (group I) at ID50 and ID100. Each of the group II lactococci and *L. lactis* BCD (group III) elicited CXCL8 production by EC but to a lesser extent as compared to *L. lactis* FnBPA-infected ECs and only at ID100 (Fig. 3; values at ID50 are not shown).

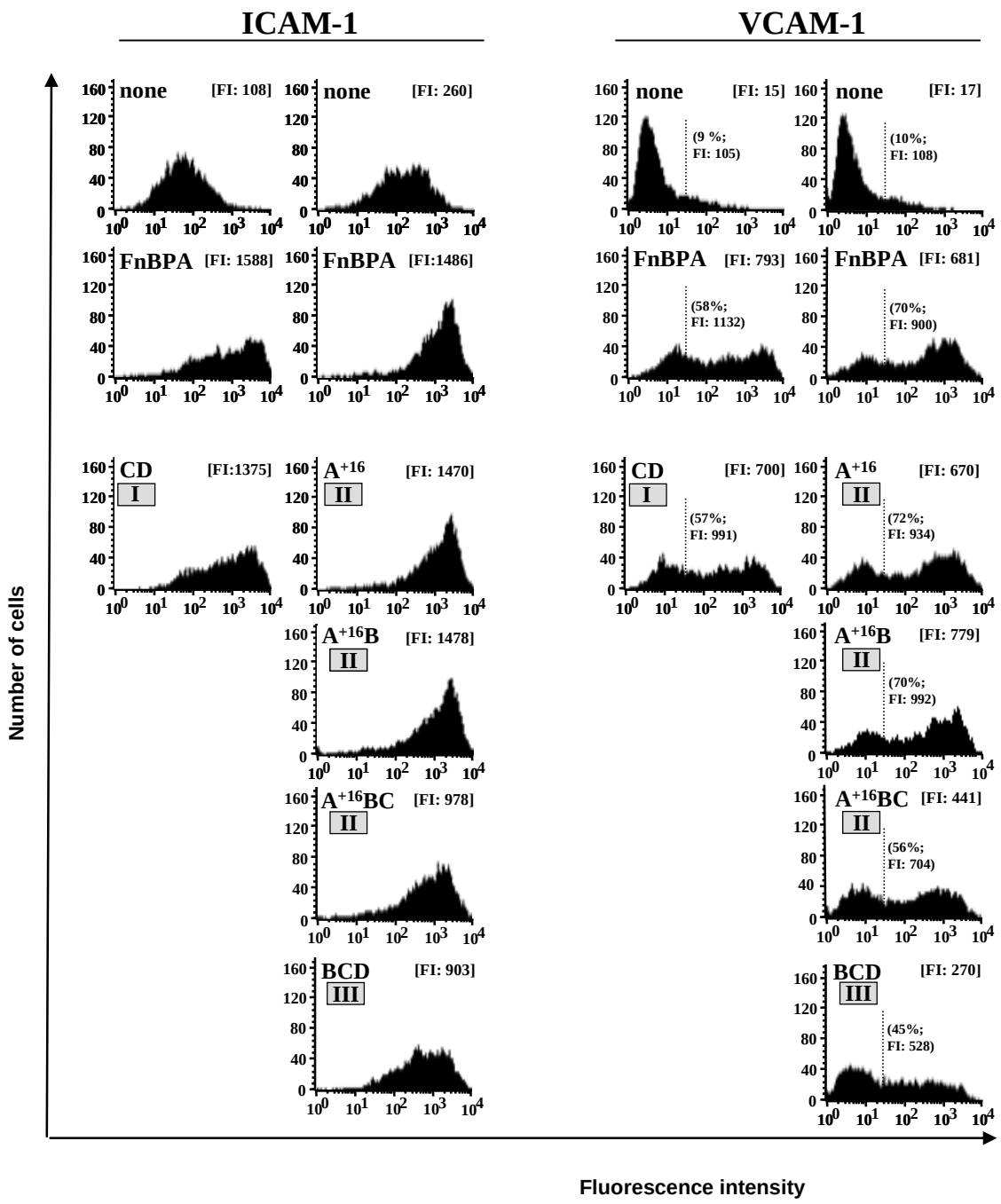

Figure 2: Flow cytometric analysis of endothelial cell surface molecule expression after infection with recombinant *L. lactis* strains.

Monolayers of 2 x 10^5 ECs were incubated for 24 h with the indicated lactococci, washed and prepared for analysis of cell surface expression of ICAM-1 and VCAM-1 by FACS. For comparison of the different strains inocula responsible for 100% infected ECs (ID100) were chosen from figure 1. Each column shows expression profiles of the same representative experiment. Values given between brackets are the mean fluorescence intensity (FI) of all cells analysed. Additionally, the proportion of ECs considered positive for expressing VCAM-1 is given in % of all cells with the associated mean FI of the positive population between parentheses.

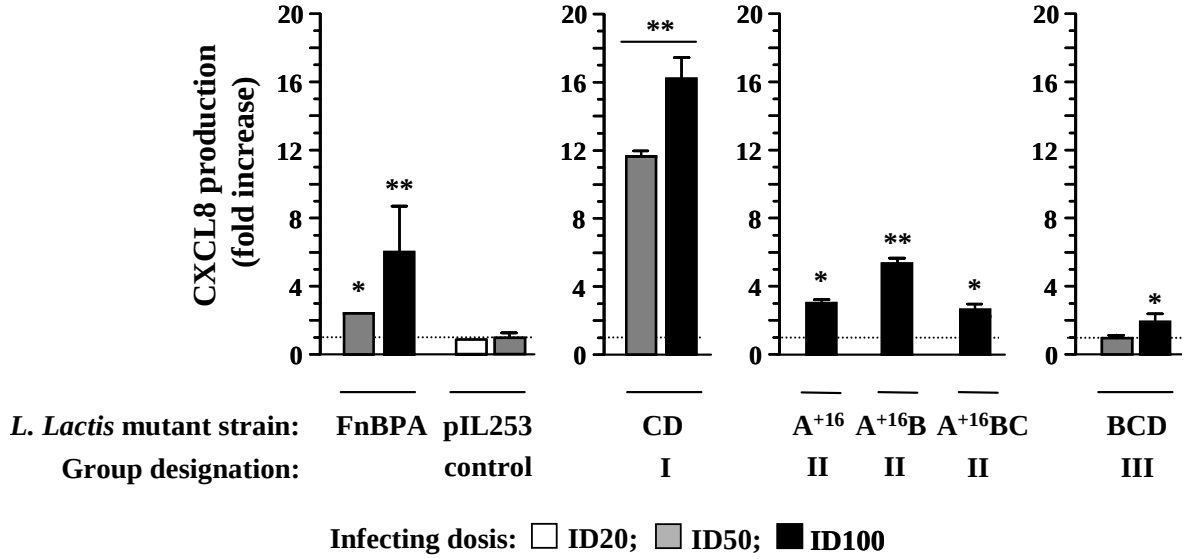

Figure 3: Endothelial CXCL8 production after infection with recombinant *L. lactis* strains.

Values of CXCL8 chemokine production were measured by ELISA from a monolayer of 2 x 10^5 ECs that had been infected for 24 h with the indicated recombinant *lactococci*. For comparison of the different strains inocula responsible for 20, 50 or 100% infected ECs (ID20, 50 or 100) were chosen from figure 1. Data are shown in fold increase of CXCL8 production of infected ECs relative to the level of control uninfected ECs (values for uninfected cells were set as 1). Data are mean ± SEM of 5 experiments with ECs from different donors. *, $P < 0.05$, **, $P < 0.01$ (paired Student's *t* test) compared to untreated cells. The grey boxes mention the group designation.

Procoagulant responses of infected ECs

We further evaluated the capacity of the various L. lactis strains to elicit a TF-dependent procoagulant activation, by measuring the EC surface expression of TF protein (Fig. 4A) as well as its biological procoagulant activity (TFA) by determining the FXa activity (Fig. 4B). Confirming our previous observations (12, 14, 19) uninfected EC cultures expressed very low levels of TF antigen on their cell surface (data not shown) and concomitantly very little TFA (7.27 ± 2.27 mU FXa / 2 x 10^5 cells, n = 6). At ID100, *L. lactis* FnBPA and *L. lactis* CD (group I) generated significantly elevated levels of TF antigen and TFA ($P < 0.01$ and $P < 0.05$ compared to uninfected ECs; n = 8; paired Student's *t* test). At ID50, however, the significantly up-regulated endothelial TF antigen expression was insufficient to result in a

significant TFA response (Fig. 4A, B). Regarding ECs infected with group II strains we observed increased expressions of TF antigen and TFA only at ID100 (Fig. 4), but not at ID50 (data not shown). ECs infected with *L. lactis* BCD (group III) at ID100 also revealed an increased TF antigen expression ($P < 0.05$ compared to uninfected ECs; n = 8; paired Student's *t* test)(Fig. 4A), but this did not coincide with TFA enhancement (Fig. 4B).

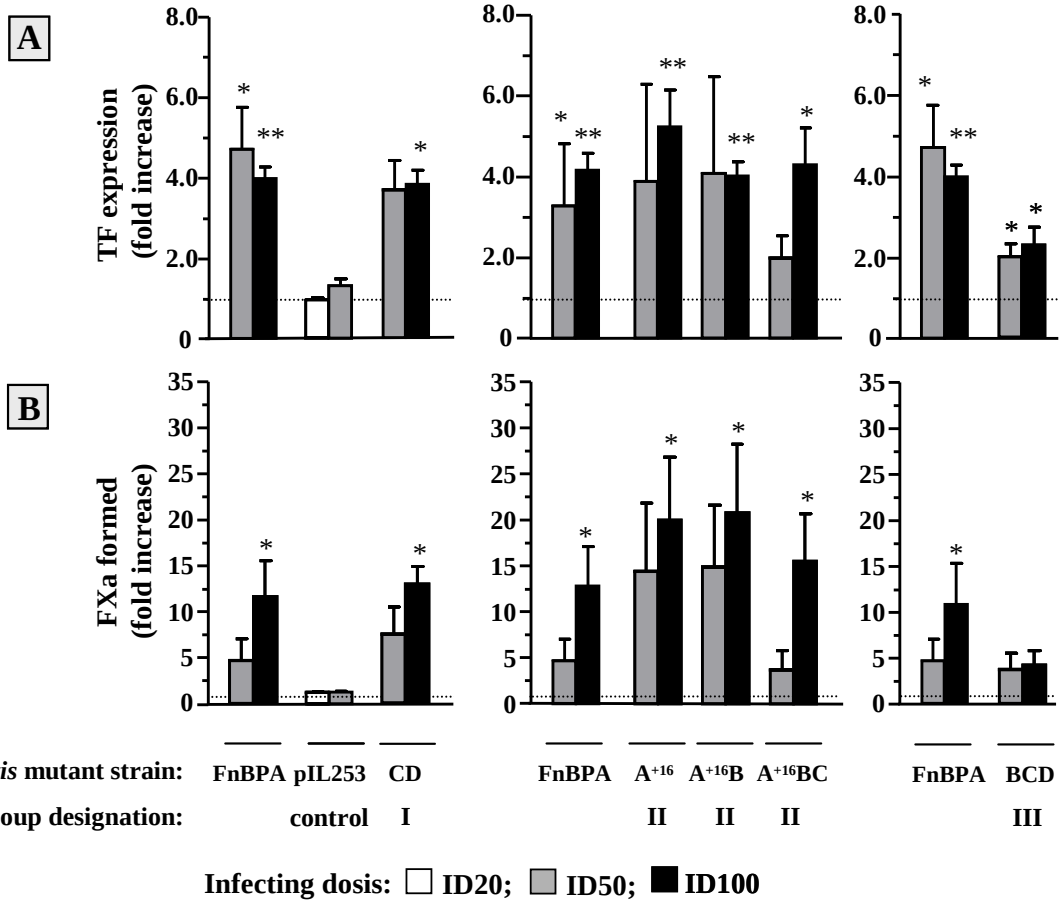

Figure 4: Procoagulant activity of ECs after infection with recombinant *L. lactis* strains.
(A) TF surface antigen expression or (B) TFA was determined on untreated ECs or ECs after incubated for 24 h with *L. lactis* mutant bacteria at ID20, 50 or 100. Data are shown in fold increase of infected ECs relative to the basal expression level of TF antigen or TFA of control untreated cells. Values for untreated cells were set as 1. TF antigen expression was determined by FACS analysis and endothelial TFA was determined by measuring FXa generation. Results shown are those of 8 (A) or 10 (B) representative experiments. *, $P < 0.05$; **, $P < 0.01$ (paired Student's *t* test) compared to untreated cells.

Subdomains of the A domain of FnBPA responsible for infection and activation of ECs

The above experiments revealed the potency of recombinant strain *L. lactis* A^{+16}, i.e., bacteria expressing of the A domain with a 16 amino acid extension in to the B domain (thus expressing FnBPA residues 1 - 559) to both infect and activate human ECs. This prompted us to further analyze which specific segment of the A domain may account for this ability. For these analyses we used the *L. lactis* recombinants that expressed constructs consisting of single or combined subdomains of the A domain, as depicted in table 1 and described in detail elsewhere (39). Monolayers of ECs were exposed for 24 h to increasing concentrations of these recombinant bacteria (from 0.5 to 50 bacteria per EC) followed by determination of the magnitude of infection and the state of activation evaluated by expression of ICAM-1 and VCAM-1. Interestingly, we found that a 127 amino acid fragment (called $A4^{+16}$ encompassing residues 432-559 of FnBPA), which spanned part of the Fg-binding module N3 as well as the first Fn-binding tandem repeat, significantly bound to immobilized Fn (Table 2) and showed a high capacity to infect ECs (Fig. 5A) that was comparable to that observed under similar experimental conditions with *L. lactis* recombinants expressing full length FnBPA (mean percentage infected cells: 92.3 % for *L. lactis* $A4^{+16}$ vs. 97.5 % for *L. lactis* FnBPA). This ability could not be further enhanced by extending the $A4^{+16}$ subdomain with combinations of other subdomains of the A domain. In addition *L. lactis* strains that lack the $A4^{+16}$ subdomain, and the first Fn-binding module in particular (i.e., *L. lactis* A1, A12 and A123) were unable to bind immobilized Fn (data not shown) and to cause significant EC infection at these bacterial concentrations (Fig. 5A).

Along with their potential to infect ECs, each of the *lactococcal* strains that expressed $A4^{+16}$, either alone or in combination with other subdomains the A domain, induced a marked increase in endothelial ICAM-1 and VCAM-1 surface expression (Fig 5B), whereas mutant strains that lack expressing of $A4^{+16}$ subdomain did not alter surface adhesion molecule expression (Fig 5B). It should be noted here that for these strains the ability to activate ECs could be analysed only at the maximal achievable level of infection, which was about 10 – 20% (indicated as ID20).

In addition we show that the *L. lactis* A4^{+16} deletion mutant strain (*L. lactis* A123B^{-16}CD) has a high probability to infect ECs and to enhance endothelial expression of cell surface adhesion molecules (Fig. 5A, B).

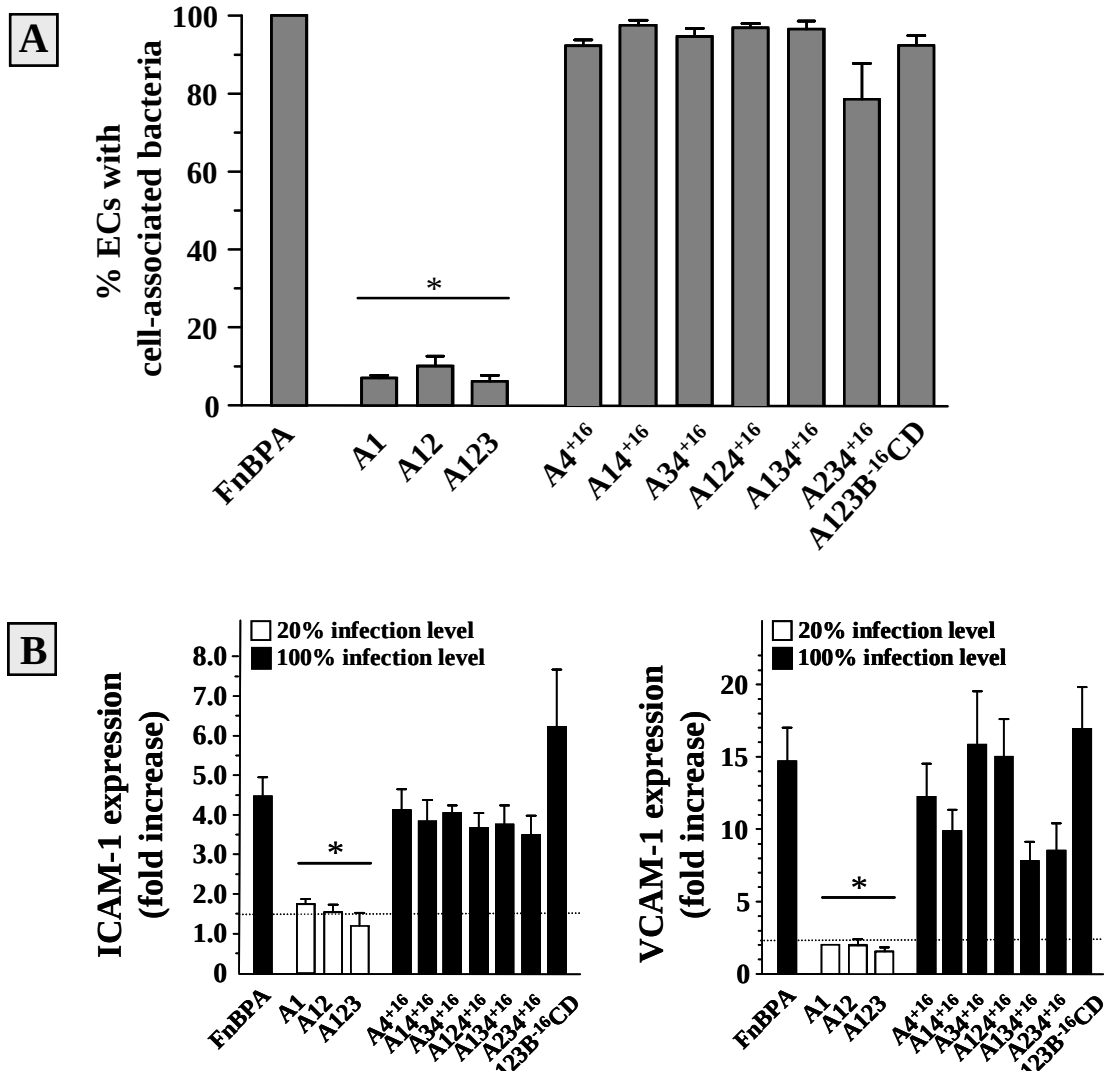

Figure 5: *L. lactis* recombinants expressing the FnBPA A4^{+16} subdomain induce endothelial infection and activation.
(A) Monolayers of 2 x 10^5 EC per well were incubated for 24 h with different inocula of the indicated *lactococcal* mutant strains, i.e., from 1 x 10^6 and 1 x 10^7 CFU per well. The percentage of infected ECs was determined after Giemsa staining. Results are expressed as mean ± SEM of at least 4 different donors relative to infection with *L. lactis* FnPBA (set to 100%). (B) Monolayers of 2 x 10^5 endothelial cells were incubated for 24 h with concentrations of the indicated lactococci to result in the maximal achievable percentage of infected ECs. For *L. lactis* A1, A12 and A123 this was about 10- 20 % (infecting dosis 20; ID20), for each of the other construct mutant strains and for *L. lactis* FnBPA this was 95 - 100% (ID100). Cell surface expression of ICAM-1 and VCAM-1 by FACS was performed in at least 4 different experiments. Data are shown in fold increase of the mean fluorescence intensity (FI) of infected ECs relative to that of control uninfected ECs. FI values for uninfected cells were set as 1.

DISCUSSION

S. aureus FnBPA is a multi-functional protein that has been shown to be sufficient to mediate binding and invasion of human ECs and subsequently to induce the typical endothelial proinflammatory and procoagulant responses that characterize a primary event in the pathogenesis *S. aureus* endovascular diseases, such as IE (12, 27).

The molecular structure of FnBPA reveals different domains. The N-terminal part of the protein is responsible for Fg- and elastin-binding (30, 31), whereas the C-terminal part binds to Fn (32-34). The traditional domain organization of FnBPA has recently been revised (see Table 1). Residues at the N-terminal of FnBPA are predicted to fold into three subdomains (N1, N2, N3), with N2 and N3 being involved in Fg and elastin binding (30, 31). The C-terminal residues responsible for Fn-binding are organized in 11 tandemly repeated Fn-binding modules (FnBr-1 to -11), each interacting with the type 1 modules at the N-terminus of Fn through a tandem beta-zipper mechanism (32, 35).

In the current study we further characterize the function of the various FnBPA subdomains in terms of EC invasion and activation. For the experiments we used a large library of constructs expressing in *L. lactis* various fragments of the staphylococcal FnBPA (39) and our in vitro model for IE, which investigates the early stage of the infection where the initial contact of intact ECs with bacteria leads to a marked increase in cell surface expression of TF and cellular adhesion molecules that in turn promote monocyte and granulocyte adhesion and monocyte-mediated TFA and EC damage (12, 19). We demonstrate that the expression of at least one Fn-binding module is necessary and sufficient to preserve its maximum potential to infect human ECs and launch EC the typical EC proinflammatory and procoagulant responses.

Classically, the Fn-binding modules were shown to be located to C-terminal part of the FnBPA protein, in the regions named C, Du, D1-D4 (33, 42). Recently, Fn-binding activity has also been described in the AB spanning region that encompasses the hinge between the C-terminal amino acids of the A domain and the first amino acids of the B1 domain (amino acids 512-550;)(34), i.e., FnBR-1 according to the recently proposed FnBPA structural organization (43). Our results confirm the importance of Fn-binding in general and of this specific domain in

particular. Indeed, we show that the expression of the C and D domains confers full Fn-binding properties to non-pathogenic *L. lactis* bacteria and that a same phenotype is also observed when lactococci express the 432-559 amino-acid residues (designated region $A4^{+16}$ in the current study).

The in parallel performed infection experiments underline the importance of these Fn-binding domains for also binding and subsequently infecting cultured human ECs. Comparative data analyses further show that the capacity of the recombinant lactococci to bind Fn correlates with the magnitude of EC infection and also activation (as will be discussed below). For example, *L. lactis* CD recombinants effectively bind Fn and exhibit a very efficient inoculum-dependent pattern of binding and infecting ECs, that was quite similar to the pattern observed with *L. lactis* expression full length FnBPA (this study, (12)), or invasive *S. aureus* strains (18, 19, 44, 45). Moreover, these results are in agreement with the observation that recombinant CD fragments fully inhibit *S. aureus*-EC interactions in various competition experiments (9).

In addition, our current data reveal that EC infection and activation by recombinant lactococci expressing FnBPA fragments lacking the C and D regions, is also observed as long as these recombinant strains express at least one Fn-binding module, especially residues 432-559 of the AB spanning region $A4^{+16}$ (i.e., *L. lactis* A^{+16}, $A4^{+16}$, AB or ABC in this study). The interaction is however less efficient as slightly higher inocula are needed to achieve maximal level of EC infection and activation. This indicates that: 1) the expression of at least one single Fn-binding region of FnBPA, under optimal infective conditions, is sufficient to confer bacterial adhesion to human ECs, and 2) any addition of Fn-binding modules, such as those included in the C and D regions, increase the avidity of Fn and EC binding and the magnitude of EC activation, as measured by their pro-inflammatory and pro-coagulant responses. This in turn decreases the inoculum necessary to achieve 100% of activation.

Moreover, in accordance with our previous study identifying *S. aureus* FnBPA as the critical staphylococcal factor sufficient for eliciting both a pro-inflammatory and a pro-coagulant EC phenotype (12), we demonstrated that the EC responses elicited by the various Fn-binding modules, is observed without the need of additional co-

factors. Our experiments also show that the degree of EC activation correlates with the quantity of bacterial-EC interactions rather than with the specific Fn-binding domain(s) involved. Apparently expression of a particular or multiple Fn-binding domains is not a pre-requisite for maximal EC activation. Indeed, once a given threshold is achieved, EC activation is maximal, as deduced from the findings that recombinant lactococci expressing one or more Fn binding domains were equally effective in eliciting activation of EC cultures when compared at an equal infection level (i.e., using an inoculum adjusted for 100% infection level). This seems to go along with the understanding that infection involves participation of Fn as a bridging molecule between staphylococcal FnBPA on the one side and endothelial Fn-binding receptors, like VLA-5, on the other side. This notion is supported by similar observations using mouse macrophages and Fn-bound *S. aureus* (46).

We do not have a clear explanation for the fact that infection with *L. lactis* CD induces higher quantities of CXCL-8 than *L. lactis* expressing full length FnBPA.

As assumed from the current results, we hypothesized that *L. lactis* BCD bacteria that express the FnBPA residues 544 – 877 encompassing the Fn-binding domain CD plus domain B, would display a full ability to infect ECs. In contrast, significantly less binding to Fn and EC with consequently less EC activation was observed. Apparently the extension of the CD domain with the B domain has led to expression of construct molecules that, e.g. by means of sterical hindrance or molecule instability, have lost their ability to optimally bind Fn or EC surfaces. A somewhat similar observation has been reported by others using FnBPA constructs that lack domain B (9). Furthermore, the finding we report on *L. lactis* BCD differs slightly from that reported earlier by Massey et al. showing that deletion of the A region had no effect on the *L. lactis* adhesion to Fn (9). Although these discrepant findings await further experimentation, a possible explanation may rely in differences in the experimental protocols. Whereas Massey et al. used relatively high numbers of bacteria to conclude on the Fn binding ability of *L. lactis* BCD, we used rather low bacterial concentrations, that were similar to the concentrations used to compare differences in the EC infection assays. It should be emphasized that in agreement with this finding, and even more important in agreement with the major conclusion of our study*, L. lactis* BCD has also less potency to infect EC and concomitantly to activate a proinflammatory and procoagulant EC response.

By repeating the experiments with *L. lactis* recombinant strains expressing single or multiple sub-regions of the A domain, we identified the amino acid residues 432-559 as essential and sufficient to confer maximal endothelial infection and activation. Co-expression of one or more different A sub-domains, such as (A1, A2 or A3) did not increase the infectivity of 432-559. Thus, confirming and extending the results of Piroth et al., FnBPA fragment $A4^{+16}$ emerges as a critical region that harbours the EC adhesive and invasive ability of the FnBPA molecule (39).

However, in contrast with the in vivo observations, identifying Fg binding as a critical pre-requisite for IE initiation (27, 39), in vitro ECs activation appears to be solely Fn-mediated. Addition or deletion of Fg-binding modules did not alter nor increase ECs activation. Synergism between various FnBPA ligand binding domains seems thus important especially for in vivo infectivity, but once the infection is established, EC activation and invasion depends mainly on Fn-binding capability.

In conclusion, this study gives further insight in the understanding of the EC activation processes induced *by S. aureus*, which is a critical step occurring in the early phase of the pathogenesis of *S. aureus* endocarditis. Our data identified the essential role of the tandemly repeated Fn-binding modules, expressed either alone or in combination to both infect and activate ECs. Moreover, we were able to show for the first time that only one of these Fn binding modules is sufficient for maximal stimulation of endothelial pro-inflammatory and pro-coagulant responses. This knowledge is important in order to develop efficient anti-adhesive treatment strategies, since each of the various Fn-binding domain should be targeted.

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CHAPTER V

Procoagulant and inflammatory responses of human endothelial cells in experimental bacterial endocarditis: Relevance of matrices used in tissue-engineering.

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ABSTRACT

In infective endocarditis (IE), bacterial metastasis involves the interaction of disseminating bacteria with endothelial cells and monocytes leading to endothelial proinflammatory and procoagulant activity. Heart valve prostheses are associated with a high risk of IE. Autologous valves constructed by matrix-based tissue engineering are under investigation to increase biocompatibility. The impact of the underlying matrices on bacterial-endothelial interactions, that characterize IE, is not known.

In the present study we compare the influence of a fibrin and collagen gel matrix on bacterial adhesion and endothelial activation. Human endothelial cells are seeded on the matrices and infected with *Staphylococcus aureus, Streptococcus sanguis* and *Staphylococcus epidermidis.*

We verified an equally high capability of *S. aureus* to infect endothelial cells seeded on the fibrin and collagen matrix compared to tissue culture plates (4.2 % and 3.7 % vs. 1.2 % of the inoculation dose; $p < 0.01$). This coincided with EC activation, cytokine secretion and surface expression of ICAM-1 and VCAM-1 and concomitant monocyte adhesion. *S. aureus* also induced a prominent tissue factor-dependent endothelial coagulation response that was not intensified by cell-bound monocytes on the gel matrices. Moderate endothelial response was seen upon infection with *S. sanguis* and *S. epidermidis* on both gel matrices.

Thus, the investigated underlying fibrin and collagen gel matrices equally increase bacterial adhesion and induce subsequent proinflammatory endothelial responses, whereas monocyte mediated tissue factor dependent coagulation decreases. Current further investigations might state the mechanisms influencing the cell-matrix interaction to evoke pathways of inflammation and fibrin deposition at the infected endovascular site.

INTRODUCTION

Infective endocarditis (IE) caused by *Staphylococcus aureus* or *Streptococcus viridans* remains a diagnostic challenge and is still associated with severe endovascular and systemic complications leading to a high mortality (1-3). Especially patients requiring heart valve prosthesis are at high risk to develop endocarditis. In IE, bacterial metastasis involves the preferential interaction of disseminating bacteria with (cardiac) vascular endothelial cells (ECs). Essential for the infection process is the species and strain dependent propensity of the microorganisms to colonize endovascular surfaces, allowing these pathogens to spread via the bloodstream to other tissues (1).

Staphylococcus aureus, *Staphylococcus epidermidis* and *Streptococcus sanguis* are among the most important bacterial pathogens responsible for endocarditis (2, 4, 5).
Upon bacterial interaction with human ECs a variety of proinflammatory endothelial responses are induced by cell surface expression of the cell adhesion proteins ICAM-1 (CD54) and VCAM-1 (CD106) mediating monocyte adhesion (6, 7) and secretion of chemokines (e.g. IL-8, MCP-1) and proinflammatory cytokines (8, 9). Moreover, endothelial tissue factor (TF) antigen expression and subsequent TF dependent coagulation is activated, which has been synergistically enhanced by adhering blood monocytes in vitro (6, 8). If ECs are important players of TF mediated coagulation in vivo is still under discussion. Increased procoagulant activity leads to formation of vegetations at the infected/injured endothelial site, seen as a critical primary feature in the early pathogenesis of IE (2). Vegetations consist of clotted fibrin, blood cells and bacteria and cause severe dysfunction of the heart valves.
Artificial or biological heart valve replacement is associated with limitations and complications, which are still of major concern. Bleeding due to required anticoagulation, limited durability and a higher risk to develop endocarditis are among the main issues (10, 11). To overrule these associated risk factors and increase the biocompatibility after heart valve implantation, autologous matrix-based tissue engineered heart valves are under investigation as patient-derived valve replacements. The patient derived matrix scaffold implicates the possibility to implant a completely autologous heart valve. Good tissue remodelling, an increased

biocompatibility and durability as well as a lower risk of developing endocarditis are expected (12-15).

Different materials are under investigation to serve as a basic matrix (16). Advances concerning structure and mechanical durability are experienced with decellularized biological scaffolds or synthetic biodegradable polymers (17-21). These materials have a number of technical and constructional drawbacks. Residual cells of decellularized scaffolds can serve as an immunological trigger and synthetic polymers lack natural attachment points.

The ideal tissue engineered heart valve may prove to be a completely autologous derivate, developed entirely from materials isolated from the patient in question. Fibrin, a major structural protein involved in the wound healing process, represents a potentially ideal cell delivery vehicle for the synthesis of completely autologous cell seeded structures because of its routine isolation from the patient's blood sample (22, 23). In the same context and in addition serving as a biodegradable scaffold, collagen based matrices are under investigation (24, 25).

The fibrin-based tissue engineered heart valve showed encouraging results in a sheep model with good tissue remodelling (22). Fibrin and collagen are known players in different inflammatory and procoagulant pathways, especially known as a potent trigger of thrombosis. The interaction of these matrices with *S. aureus* and other bacterial agents is not known yet, but the potential role of these newly constructed heart valves, and especially their matrix, in the pathogenesis of endovascular infection seems an important issue.

Recently published studies evaluated the potential of inflammation and thrombogenicity of biological cardiovascular scaffolds. If the matrices inherit an underlying co-stimulating factor in terms of inducing endocarditis is not known yet (26-30).

Therefore, this study was designed to investigate the early steps in the pathogenesis of endovascular infection in a matrix based, in vitro model of endocarditis. Bacterial adhesion, consecutive endothelial proinflammatory (ICAM-1, VCAM-1, IL-8 production) and procoagulant activity (TF-activity) as well as monocyte adhesion were evaluated. Endothelial cells seeded on matrices served as the base of the experiments investigating if the matrix used in tissue engineered heart valves has an influence on bacterial-endothelial interaction.

MATERIALS AND METHODS

Reagents

Trypsin-EDTA was purchased from GIBCO Laboratories (Grand Island, USA), RPMI 1640 and fetal calf serum (FCS) from Biochrom (Berlin, Germany). Collagenase type 1A was from Roche (Mannheim, Germany), bovine serum albumin (BSA) from Sigma-Aldrich Inc. (St. Louis, Mo., USA), gelatin from Serva (Heidelberg, Germany) and glutaraldehyde from Polyscience Inc. (Warrington, UK). Human serum was used from Sigma-Aldrich Inc. (St. Louis, Mo., USA) and inactivated at 56°C for 30 minutes (HuSi).

Clotting factor X, factor VII and the chromogenic substrate PefachromeFXa® were obtained from Kordia (Leiden, The Netherlands). Acetic acid, $CaCl_2$ and TRIS-base came from Merck (Darmstadt, Germany).

Bacterial strains and growth conditions

The bacteria used in this study were *S. aureus* 42D, *S. sanguis* NCTC 7864 and *S. epidermidis* ATCC 149900 (9). Bacterial suspensions were stored at – 70°C. For use in the infection assays, *S. aureus* was grown overnight without shaking in routine Nutrient Broth II (NB) and *S. epidermidis* and *S. sanguis* in Todd-Hewitt broth (THB) at 37°C. Then all bacteria were opsonized for 30 min at 4 rpm in M199 containing 0.1 % (w/v) gelatin and 10 % fresh human serum and diluted to the appropriate concentration in medium 199 (M199; GIBCO Laboratories, Grand Island, USA) containing 10 % heat inactivated (30 min, 56°C) human serum (HuSi) prior to use in the infection assay.

Fibrin gel composition

Human fibrinogen (plasminogen free; Sigma, Seelze, Germany) was dissolved in purified water and dialysed with a cut-off membrane (Novodirect, Kehl, Germany) of 6000 – 8000 molecular weight overnight against Tris-buffered saline (TBS). The fibrinogen concentration following sterile filtration was estimated by measuring absorbance at 280 nm with a spectrophotometer Beckman Coulter Du640B (Beckman, Fullerton, USA) with a molar extinction coefficient of 1.5. The final concentration of the fibrin solution was adjusted to 8 mg/ml with TBS. Gel

polymerisation was initialized by adding thrombin solution (40 U/ml) and 50 mM $CaCl_2$ in TBS.

Collagen gel composition

This matrix is based on collagen type-I supplied as an aqueous solution of 6 mg/ml in 0.1 % acetic acid (Arthro Kinetics, Esslingen, Germany). Collagen solution remained liquid when stored at 4°C. Before use, the collagen solution was centrifuged at 700g for 10 min and diluted 1:1 by a neutralisation buffer (2 x DMEM/2M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (0.93/0.07)). Gel polymerisation was achieved by transferring the gel to room temperature.

Endothelial cell outgrowth on the different matrices

To compare the density of the endothelial cell layer on the different matrices nuclear staining with DAPI (4'-6-diamidino-2-phenylindole) was performed. Cells were washed with PBS and fixated for 15 min at room temperature in 4% paraformaldehyde. Thereafter ECs were incubated for 5 min with DAPI (1:5000) resolved in a solution of PBS containing saponine 2%. Cell nuclei were counted by fluorescence microscopy (Axiovert 200M, Carl Zeiss, Jena, Germany) at 500 nm.

To visualize the EC outgrowth on the gel matrices, ECs were seeded on fibrin gel with a density of 1 x 10^5 cells / cm^2. Seeded gels were fixed in carnoy, dehydrated and embedded in paraffin. Cut sections of the seeded gels were subsequently stained with hematoxylin and eosin (H&E) to visualize the EC monolayer on the surface of the gels and exclude cell migration into the gels. Sections were analyzed using routine bright field light microscopy (AxioImager D1; Zeiss, Jena, Germany), and images were acquired using a digital colour camera (AxioCam MRc; Zeiss, Jena, Germany).

Preparation of EC and monocyte cultures

ECs were taken from the human great saphenous vein from patients receiving a cardiac bypass operation. After approval by the Human Ethical Committee of the Duesseldorf University Hospital and informed consent of patients ECs were isolated and cultured on gelatin-coated tissue culture dishes in endothelial cell basal medium added by supplement pack/endothelial cell growth medium (PromoCell, Heidelberg,

Germany) and 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 100 U/ml amphothericin-B in a 5 % CO_2 incubator at 37°C, as described elsewhere (7). Monolayers of second-passage ECs grown on fibrin or collagen gel, yielding a minimal cell density of 800 ECs /mm^2, were used in the experiments. ECs grown on gelatin-coated tissue plates were taken as a control.

The THP-1 human monocytic cell line was maintained in RPMI 1640 containing 100 U/ml penicillin G, 0.1 mg/ml streptomycin and 10 % FCSi. We showed previously that THP-1 cells expressed the surface adhesion molecules required for monocyte-EC interaction and produced tissue factor activity (TFA) on appropriate stimulation (6, 8). Prior to their use in the experiments THP-1 cells were resuspended in M199 containing 5 % HuSi, 5 % FCSi and 5 µg/ml erythromycin. Throughout the manuscript THP-1 cells are mentioned as monocytes.

EC infection assay

Confluent monolayers of ECs on the different matrices were incubated under variable experimental conditions with opsonized *S. aureus* 42D, *S. sanguis* NCTC 7864 and *S. epidermidis* ATCC 149900 at 37°C. Subsequently, the monolayers were washed three times with warm[1] PBS to remove the bacteria that were not associated with the ECs. ECs were detached from the matrices using collagenase 0.5% for 20 min and subsequently in a second step by sonication. The number of cell-adherent and cell-invaded bacteria was determined by colony counts after plating serial dilutions of the lysate on agar plates and overnight incubation at 37°C. The number of bacteria used in the infection assay was also confirmed afterwards by colony counts. Results were expressed as percentage of the inoculation dose.

For each bacterium two concentrations of inocula were investigated in the assays on a monolayer of about 2 x 10^5 ECs: Concentrations used for *S. aureus* were 1 x 10^6 and 1 x 10^7 and for *S. sanguis* and *S. epidermidis* 5 x 10^6 and 5 x 10^7 bacteria. There was no difference found concerning the concentrations used. Therefore data obtained with the lower concentrations are not shown.

[1] PBS at 37°C was chosen to be compatible with the underlying EC culture.

Flow-cytometric analysis of EC surface molecules

EC monolayers were infected for 1 h with variable numbers of opsonized bacteria, washed with PBS and cultured for an additional period of 23 h. The cells were harvested using collagenase 0.5 %, washed and collected in cold PBS with 0.1 % FCSi (wash buffer) and taken under three incubation steps on ice: 15 min with PBS containing 1 % goat serum, 30 min with 1 µg/ml of the appropriate mAb and 30 min with phycoerythrin-conjugated goat-anti-mouse Ig (Southern Biotechnology Associates Inc. Birmingham, USA). In between each step cells were washed with cold washing buffer. At least 5000 cells were analyzed by flow cytometry (Becton Dickinson FACSCaliber, Franklin Lakes, USA) using CellQuest® software. The following mouse mAb against surface molecules of human ECs or monocytes were used: anti-CD54 (ICAM-1) mAb 15.2 (Santa Cruz Biotechnology, Santa Cruz, USA); anti-CD106 (VCAM-1) mAb 1G11B1 (Biosource International, Camarillo, USA) anti-CD142 (TF) mAb HTF-1 (Becton Dickinson, Franklin Lakes, USA) and for control cells anti-$\alpha_5ß_1$ (Biosource International, Camarillo, USA).

Determination of cytokine production

Supernatants from ECs cultured on the different matrices and infected with the above mentioned bacteria were assayed for production of the chemokines proinflammatory cytokines IL-6 and IL-8 and monocyte chemoattractant protein (MCP-1) using the PeliKine Compact™ human IL-8 enzyme-linked immunosorbent assay kit (Sanquin, Amsterdam, The Netherlands) and Cytoset™ immunoassay kits for human IL-6 and human MCP-1 (Biosource International, Camarillo, USA). The assays were performed according to the supplier's instructions. Limits for detection of IL-8, IL-6 and MCP-1 were 8.0 pg/ml, 50.0 pg/ml and 30.0 pg/ml respectively.

Monocyte-EC adhesion and coculture conditions

About 1.5 x 10^5 monocytes in M199 containing 10 % HuSi were colored with BCECF (2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein) and added to monolayers of approximately 2 x 10^5 bacteria-infected ECs grown on the different matrices and subsequently cocultured for 1 or 6 h at 37 °C in a 5 % CO_2 incubator.

The 1 h time point was included because it is an optimal time point to determine immediate effects of EC activation on monocyte adhesion (7, 31), the 6 h time point being optimal for determination of the total TFA in these cocultures (6). After coculture the EC layers were washed 5 times with warm PBS to remove non-adherent monocytes. The remaining cells were fixed with methanol for 15 minutes. The number of adherent monocytes was quantified by photometric analysis using a Tecan Infinite M200 photometer (Tecan, Crailsheim, Germany). Standard values of defined monocyte concentrations were taken along to calculate a standard curve.

Analysis of TF-dependent procoagulant activity (TFA)

TFA was measured by transformation of clotting factor X (FX) to FXa as described earlier (32). Following infection and/or coculture with monocytes, TFA of EC monolayers was investigated after allowing formation of TF/FVII/Ca^{2+} complex. Transformation of clotting factor X (FX) to FXa was marked by using PefachromeFXa® (Kordia, Leiden, The Netherlands) as the chromogenic substrate of FXa. FXa concentrations were calculated using a calibration curve of purified FX that was fully activated with Russel Viper Venom (Chromogenix, Mölndal, Sweden). Values were calculated as mU FXa / well containing about 2 x 10^5 ECs and in some experiments variable numbers of endothelial cell-bound monocytes. Figures show results as fold increase values compared to unstimulated cells on the same matrix.

Statistical analysis

Data were analyzed with the PASW program for Windows software, version 18 (SPSS GmbH Software, Muenchen, Germany). The paired Student's *t*-test (two-tailed) was used to control for variation in measurements due to the fact that for each experiment ECs isolated from different donors were used. Results are expressed by the mean value ± SEM. Values of $p \leq 0.05$ were considered significant.

RESULTS

Outgrowth of the endothelial monolayer on the different matrices

The endothelial cell layer was investigated for its density by staining the cell nuclei with DAPI. Endothelial cell outgrowth showed equal cell counts per cm^2 on all matrices. We found 1.14 x 10^5 cells / cm^2 on fibrin gel, 1.04 x 10^5 cells / cm^2 on collagen gel and 1.02 x 10^5 cells / cm^2 on tissue culture plates.

ECs were verified to construct an intact monolayer on the surface of the gel matrices without any cell migration into the gel as shown by HE staining on fibrin gel in Figure 1.

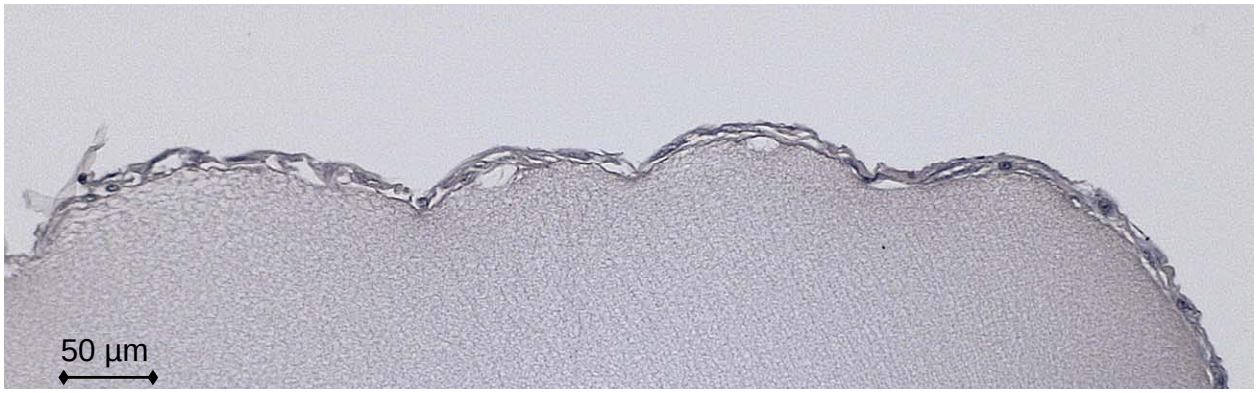

Figure 1: Endothelial cell outgrowth on fibrin gel.
ECs were stained with HE after being seeded at a density of 2 x 10^5 cells / cm^2 on fibrin gel. An intact EC monolayer is shown on the gel surface without any cell migration into the gel.

Infection of ECs seeded on the fibrin and collagen matrix after bacterial incubation

To determine if a basic matrix used in tissue-engineering influences bacterial attachment of different species belonging to the main causes of bacterial endocarditis, monolayers of ~ 2 x 10^5 ECs were seeded on fibrin gel, collagen gel and tissue culture plates as a control surface. ECs grown on the matrices were exposed for 1 h to opsonized *S. aureus*, *S. sanguis* and *S. epidermidis*.

S. aureus exhibited a very efficient, similar pattern of infecting ECs seeded on the two gel matrices (Fig. 2) with 3.7 – 4.5 % of the inoculated bacteria measured in the infection assay. In contrast, ECs seeded on tissue culture plates showed a significant

lower degree of bacterial adhesion compared to the gel matrices with 1.5 % of the inoculation dose found in the assay (Fig. 2, $p < 0.01$, paired *t*-test, n = 9).

Both, *S. sanguis* and *S. epidermidis* were less potent to infect ECs. There was no difference concerning the degree of infection comparing the two gel matrices with 0.6 – 1.3 % of the inoculation dose found. The infection level was even significantly lower after incubation with *S. sanguis* and *S. epidermidis* when of ECs were grown on tissue culture plates (Fig. 2, $p < 0.05$, paired *t*-test, n = 9).

Exposure of ECs to the different bacteria did not result in cell detachment or monolayer destruction. Bacterial outgrowth in the medium was negligible during 1h (data not shown).

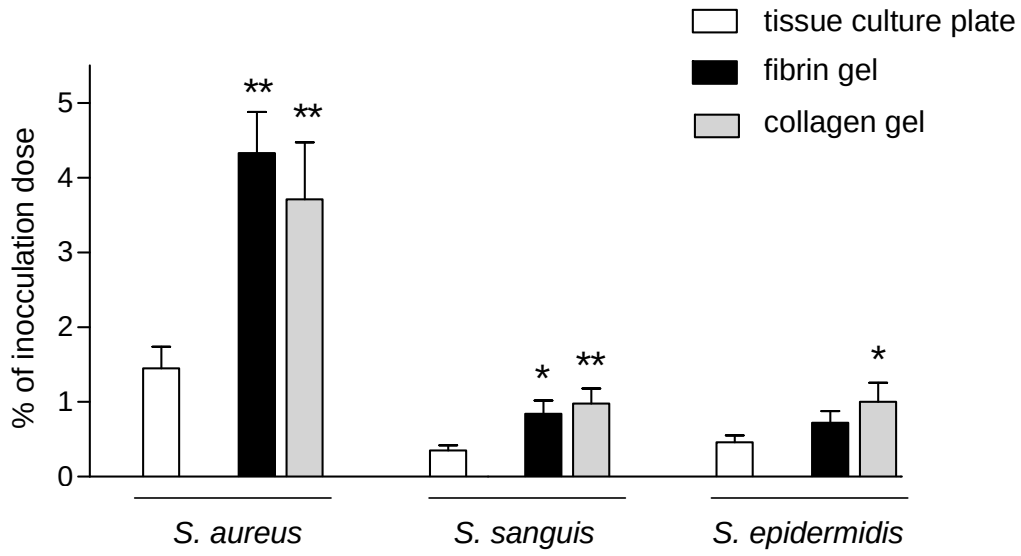

Figure 2: Bacterial challenge associated with ECs seeded on the matrices.
EC monolayers (2 x 10^5 EC/well) on the different matrices were incubated for 1 h with 1 x 10^7 *S. aureus*, and 5 x 10^7 *S. sanguis* and *S. epidermidis*, washed and detached from the matrices. Number of adherent bacteria was determined by colony counts. Results are expressed as percentage of the inoculation dose. Data are mean ± SEM of 9 experiments with ECs from different donors. Paired *t*-test: * = $p < 0.05$; ** = $p < 0.01$ gel matrix compared to condition on tissue culture plates.

Bacterial adhesion on plain matrices

Secondly, bacterial strains were investigated for their direct adherence to the fibrin and collagen gel matrix without pre-seeded endothelial cells. Incubation with *S. aureus* verified a higher adhesion rate on the two gel matrices compared to the tissue culture plates, which was significant for fibrin gel ($p < 0.01$, paired *t*-test, n = 4),

respectively. *S. aureus* adherence did not significantly differ when comparing the two gels matrices.

Incubation of *S. sanguis* and *S. epidermidis* on the two gel matrices led to very low adhesion capacities (Table 1).

Infection on matrices without ECs (% of inocculation dose)			
	cell culture plate (MEAN ± SEM)	**fibrin gel** (MEAN ± SEM)	**collagen gel** (MEAN ± SEM)
S. aureus	0.32 (± 0.10)	5.66 (± 0.45) **	2.33 (± 1.48)
S. sanguis	0.06 (± 0.02)	0.53 (± 0.02)	0.17 (± 0.11)
S. epidermidis	0.26 (± 0.03)	0.42 (± 0.21)	0.33 (± 0.01) **

Table 1: Bacterial adhesion on matrices without ECs cultured.
Paired *t*-test : ** = $p < 0.01$ gel matrix compared to condition on tissue culture plates

Proinflammatory activation of ECs seeded on different matrices upon bacterial infection

Next we explored whether the infection with the different strains elicited EC activation by evaluating surface expression of ICAM-1 and VCAM-1 and production of IL-6, IL-8 and MCP-1 as proinflammatory factors related to endovascular infections.

Adhesion molecule expression

Uninfected control cells were evaluated for their basic expression of the integrin $\alpha_5ß_1$ on the different matrices and showed similar values of a MFI ± SEM of 193.4 ± 26.9 on fibrin gel, 260.1± 29.1 on collagen gel and 206.3 ± 32.7 on tissue culture plates (not significant, paired *t*-test, n = 4).

Uninfected ECs express moderate constitutive levels of ICAM-1 on the different matrices which were significantly lower when ECs were cultured in the gel matrices

compared to ECs cultured on tissue culture plates (MFI ± SEM of 156.3 ± 21.2 on fibrin gel, 181.2 ± 22.4 on collagen gel and 234.7 ± 21.4 on tissue culture plates; $p < 0.05$, paired *t*-test, n = 8). Uninfected ECs express very low levels of VCAM-1 on the different matrices which were significantly higher in ECs cultured on collagen gel compared to fibrin gel ($p < 0.05$, paired *t*-test, n = 8) and tissue culture plates (MFI ± SEM of 14.6 ± 1.8 on fibrin gel, 17.6 ± 1.1 on collagen gel and 13.5 ± 1.6 on tissue culture plates; $p < 0.01$, paired *t*-test, n = 8) molecules on their cell surface. Basic levels were set to 1 in figures 3A and 3B.

Endothelial infection with *S. aureus* was accompanied by a marked increase in surface expression of ICAM-1 and VCAM-1 (Fig. 3) and reached levels quite similar to that obtained after incubation with 5 µg/ml IL-1, a potent stimulator of endothelial proinflammatory responses (6, 9), which amounted to a 3.3 - 3.9 fold and 1.5 – 2.7 fold increase for ICAM-1 and VCAM-1, respectively, after 24 h stimulation on the different matrices.

Values achieved for ECs cultured on fibrin and collagen gel were not significantly different, but *S. aureus* infected ECs on fibrin gel showed a slightly higher ICAM-1 expression when compared to ECs seeded on tissue culture plates ($p < 0.05$, paired *t*-test, n = 8).

Adhesion molecules show a moderate elevation after infection with *S. sanguis* and *S. epidermidis* (Fig. 3), despite the fact that low infection levels are seen at these concentrations, as shown in figure 2. Measured ICAM-1 and VCAM-1 values do not differ between the fibrin and collagen gel matrix.

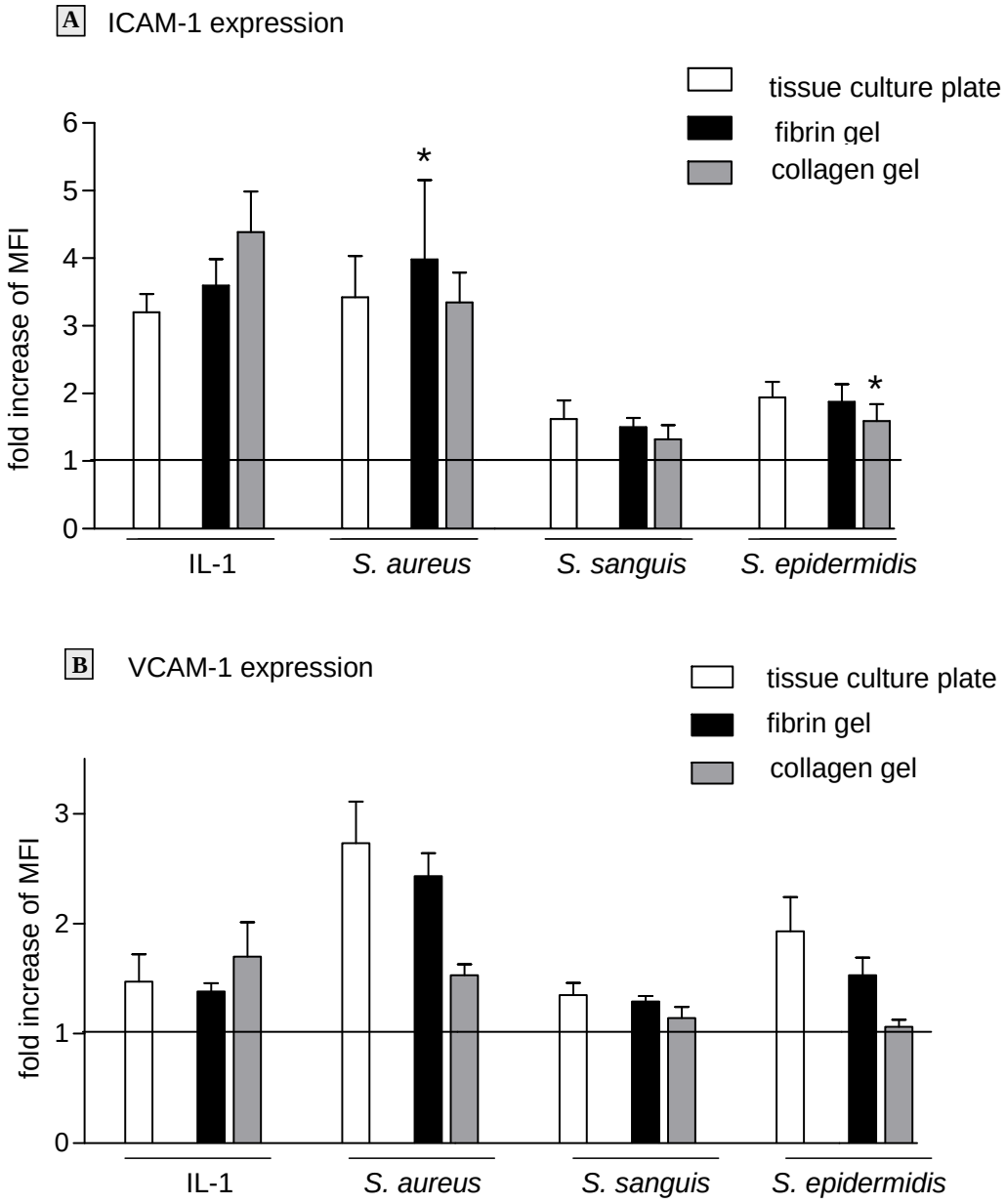

Figure 3 A and B: Flow cytometric analysis of endothelial ICAM-1 and VCAM-1 expression after bacterial challenge.
Monolayers of 2 x 10^5 ECs on the different matrices were incubated for 1 h with 1 x 10^7 *S. aureus*, and 5 x 10^7 *S. sanguis* and *S. epidermidis*, washed, cultured for an additional period of 23 h and prepared for analysis of cell surface expression of ICAM-1 (A) and VCAM-1 (B) by FACS. Data are shown in fold increase of the mean fluorescence intensity (MFI) of infected ECs relative to the level of ICAM-1 and VCAM-1 expression on control uninfected ECs (MFI values for uninfected cells were set as 1). Paired *t*-test: * = $p < 0.05$ gel matrix compared to condition on tissue culture plates.

Cytokine production

Uninfected ECs secreted moderate amounts of IL-8 and MCP-1 (1.73 – 2.35 ng/ml for IL-8 and 1.33 – 2.61 ng/ml for MCP-1) and negligible amounts of IL-6 per monolayer of 2 x 10^5 ECs seeded on the different matrices (Fig. 4).

Endothelial production of IL-8 and MCP-1 was markedly induced by *S. aureus* and to a lesser degree by *S. sanguis* and *S. epidermidis* on all matrices, whereas IL-1 stimulation led to maximal cytokine production (Fig. 4).

Interestingly, a significant lower quantity of IL-8 was produced by ECs seeded on collagen gel compared to fibrin gel after bacterial incubation (*S. aureus:* $p < 0.05$; *S. sanguis* and *S. epidermidis:* $p < 0.01$, paired *t*-test, n = 4). MCP-1 was also produced to a lesser extend by *S. aureus* stimulated ECs seeded on collagen gel vs fibrin gel ($p < 0.05$, paired *t*-test, n = 4).

Elicited MCP-1 production in unstimulated ECs as well as upon infection with *S. aureus* and *S. sanguis* ($p < 0.05$, paired *t*-test, n = 4) with ECs seeded on collagen gel was lower compared to ECs grown on tissue culture plates.

In contrast, none of the bacteria but IL-1 induced relevant endothelial production of the inflammatory cytokine IL-6 (Fig. 4). Achieved IL-6 production upon IL-1 incubation was lower when ECs were seeded on the two gel matrices compared to tissue culture plates ($p < 0.05$, paired *t*-test, n = 4).

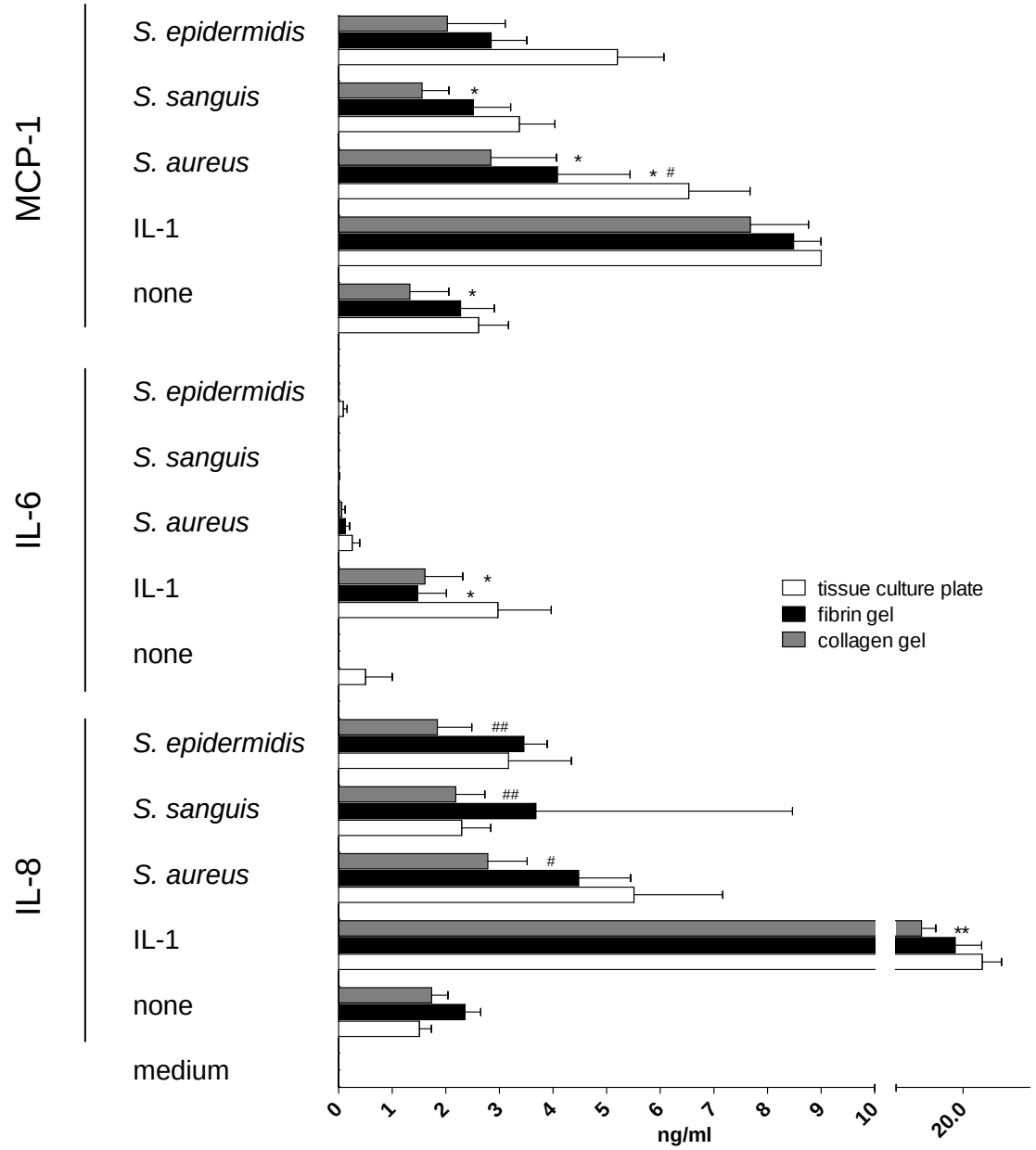

Figure 4: Endothelial IL-8, MCP-1 and IL-6 production after bacterial challenge.
Values of IL-8, MCP-1 and IL-6 protein production were measured from a monolayer of 2 x 10^5 ECs seeded on the different matrices. The amount of cytokine secreted by a monolayer of ECs 23 h after 1 h-infection with 1 x 10^7 *S. aureus*, and 5 x 10^7 *S. sanguis* and *S. epidermidis* was determined by ELISA. Data are mean ± SEM of 4 experiments with ECs from different donors. None represents basal cytokine production of uninfected cells.
Paired *t*-test: * = $p < 0.05$; ** = $p < 0.01$ gel matrix compared to condition on tissue culture plates, # = $p < 0.05$; ## = $p < 0.01$ collagen compared to fibrin gel.

Monocyte adhesion to ECs seeded on different matrices upon bacterial infection

Infection with *S. aureus* resulted in a pronounced increase of EC-bound monocytes of about 30 – 60% within 6 h of coculture on all matrices (Fig. 5). This percentage is comparable with that obtained with monolayers of IL-1 stimulated ECs (Fig. 5) and also resembles our previously published values for *S. aureus* infected ECs (6). Interestingly, monocyte adhesion to ECs seeded on the two gel matrices was significantly higher compared to ECs seeded on tissue culture plates upon incubation with IL-1, *S. aureus* and *S. sanguis* ($p < 0.01$, paired *t*-test, n = 10). Control ECs and *S. epidermidis* infected ECs showed a higher percentage of monocyte adhesion when seeded on fibrin gel compared to tissue culture plates ($p < 0.05$, paired *t*-test, n = 10). There was no relevant difference in monocyte adhesion comparing the fibrin and collagen gel.

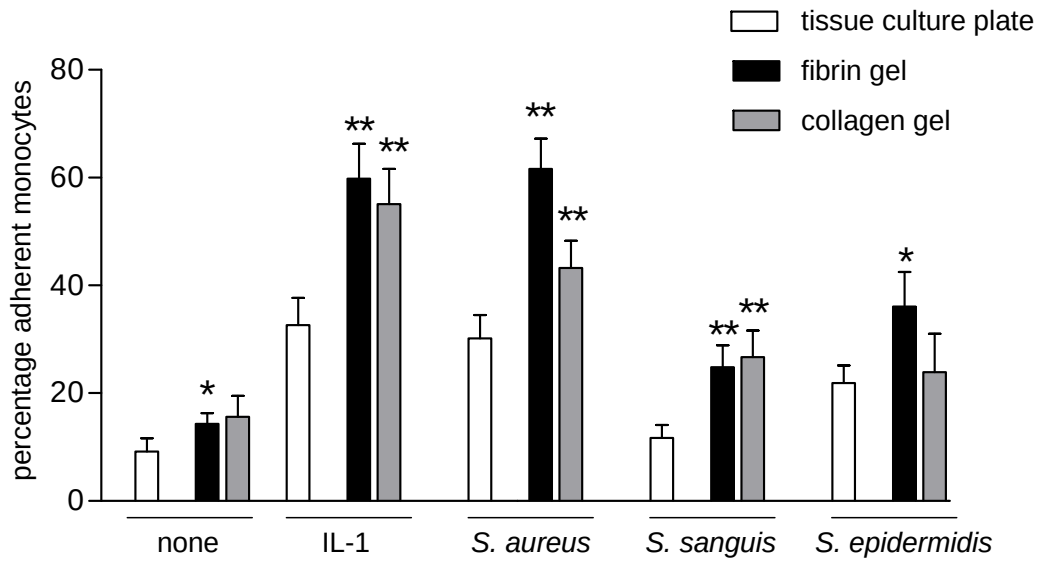

Figure 5: Monocyte adhesion to ECs after bacterial challenge.
EC monolayers were incubated for 24 h with medium alone (untreated cells; none) or medium containing 5 ng/ml IL-1, 1 x 10^7 *S. aureus*, and 5 x 10^7 *S. sanguis* and *S. epidermidis*. After washing, stained monocytes were added and cocultured with the ECs. At 6 h during coculture the number of EC-associated monocytes was determined. Results shown are those of 10 representative experiments. Paired *t*-test: * = $p < 0.05$; ** = $p < 0.01$ gel matrix compared to condition on tissue culture plates.

Procoagulant activity of ECs seeded on different matrices upon bacterial infection

The above experiments demonstrate an importance of the basic matrix used for endothelial seeding concerning activation of proinflammatory endothelial responses. To further explore the activating potential of the matrices we measured the endothelial TF-dependent procoagulant response to bacterial infection.

EC procoagulant activity was investigated by measuring the biological activity of TF protein performing a TF-dependent procoagulant activity (TFA) assay (Fig. 6).

Uninfected ECs from different donors showed a high degree of variation in their TFA-response to activating stimuli. To circumvent this problem the data are expressed as fold increase in TFA as compared to level in uninfected cells of the same donor (Fig. 6). Uninfected ECs expressed similar low TFA on the different matrices (tissue culture plate 2.88 ± 0.32; fibrin gel 2.84 ± 0.42; collagen gel 3.13 ± 0.55 mU FXa/2 x 10^5 cells, not significant, paired *t*-test, n = 10).

A marked increase in TFA was found after IL-1 stimulation and infection with *S. aureus* as well as to a lesser degree upon contact with *S. sanguis* and *S. epidermidis* on tissue culture plates. TFA levels reached by ECs seeded on the two gel matrices are slightly higher upon bacterial contact compared to IL-1 stimulation. Overall values were not significantly different between the two gel matrices except for *S. sanguis* ($p < 0.05$; paired *t*-test, n = 10).

Procoagulant activity of ECs seeded on different matrices upon bacterial infection during coculture with monocytes

S. aureus infection of ECs results in monocyte recruitment and monocyte-dependent amplification of the endothelial TF-dependent coagulation response, as previously shown (4, 6, 8, 9). The same in vitro model of experimental endocarditis was used to evaluate the contributing potential of the basic matrices in these events. Infected EC monolayers were cocultured with monocytes on the different matrices and the total level of TFA generated by the cocultured cells was evaluated.

Uninfected ECs in coculture with monocytes expressed significantly lower basic TFA on the gel matrices compared to tissue culture plates (tissue culture plate 8.86 ±

1.94; fibrin gel 3.54 ± 0.37; collagen gel 3.92 ± 0.56 mU FXa/2 x 10^5 cells, $p < 0.05$, paired *t*-test, n = 8).

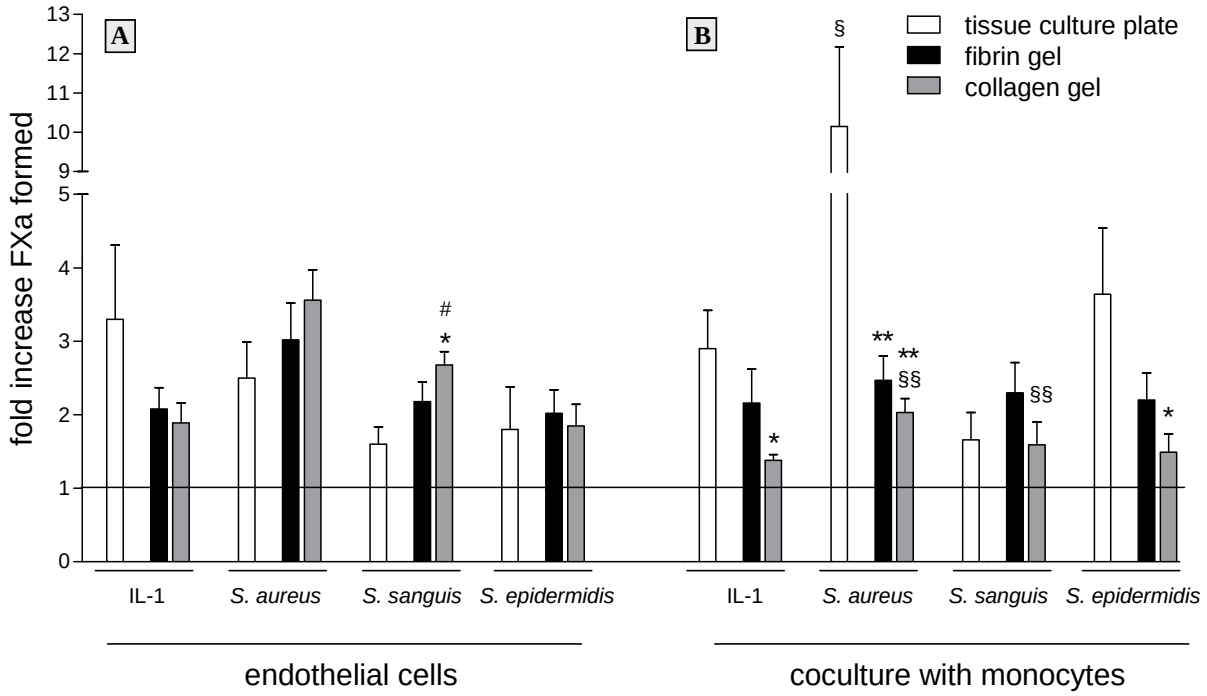

Figure 6 A and B: Endothelial TFA and monocyte-dependent augmentation of TFA during coculture of monocytes with ECs after bacterial challenge.
(A) Endothelial TFA was determined 24 h after treatment with IL-1, 1 x 10^7 *S. aureus*, and 5 x 10^7 *S. sanguis* and *S. epidermidis* measuring FXa generation. Data are expressed as fold increase relative to the basal TFA of untreated ECs. Results shown are those of 10 representative experiments.
(B) EC monolayers were incubated for 24 h with medium alone (untreated cells; none) or medium containing 5 ng/ml IL-1, 1 x 10^7 *S. aureus*, and 5 x 10^7 *S. sanguis* and *S. epidermidis*. After washing, monocytes were added and cocultured with the ECs. After 6 h the amount of FXa released by the cells in coculture was measured by the TFA-assay. Results shown are those of 8 representative experiments. Paired *t*-test: * = $p < 0.05$; ** = $p < 0.01$ gel matrix compared to condition on tissue culture plates; # = $p < 0.05$ collagen compared to fibrin gel; §§ = $p < 0.01$ compared to the respective condition without monocytes in Fig. 6A.

Upon infection with *S. aureus* a maximal increase of TFA was induced after coculture with monocytes on tissue culture plates (Fig. 6B), which was about 10-fold higher ($p < 0.01$, paired *t*-test, n = 8) than amounts of FXa generated in the absence of monocytes depicted in Fig. 6A. Therefore, ability of *S. aureus* infected ECs to avidly bind monocytes (Fig. 5) coincided with a significant increase in the amount of FXa

generated during subsequent coculture of the cells (Fig. 6B). Interestingly, this marked increase of TFA was found only on tissue culture plates.

In contrast, the coculture of monocytes on the two gel matrices did not coincide with such TFA enhancement ($p < 0.01$, paired *t*-test, n = 8, Fig. 6B). Therefore, the significant increase in monocyte adhesion to *S. aureus*-infected ECs (Fig. 5) did not lead to a subsequent induction of TFA during coculture with monocytes on fibrin and collagen gel.

TFA in IL-1-activated ECs remained unchanged compared to the values reached by ECs alone on all matrices which goes along with previous findings (6).

The coculture of monocytes with *S. sanguis* and *S. epidermidis*-infected ECs, a condition that did not lead to relevant EC infection (Fig. 2), resulted in a moderate level of TFA that was not different comparing the two gel matrices. *S. epidermidis*-infected ECs on collagen gel showed lower values when compared to ECs cultured on tissue culture plates ($p < 0.05$, paired *t*-test, n = 8) and *S. sanguis*–infected ECs induced even lower TFA levels when compared to the ECs without monocytes involved (Fig. 6B). Apparently, the presence of relatively low numbers of monocytes bound to the surface of *S. sanguis* and *S. epidermidis*-infected ECs (11 - 36 %; Fig. 5) is sufficient to elicit a TFA response in the cocultured cells.

DISCUSSION

In the present study we investigate a fibrin and collagen matrix used in tissue-engineering and study its implication in eliciting a proinflammatory and procoagulant phenotype of intact human ECs that typifies an essential initial phase in the early stage of endovascular infection, such as IE (6-9).

The use of bioresorbable scaffolds, accellular xenografts and collagen- or fibrin-based scaffolds remodelled by cells to resemble a valve leaflet is an attractive approach to tissue engineering (23, 33, 34). Both, fibrin and collagen matrices have been shown to serve as a biological, three-dimensional matrix facilitating seeded cells to maintain viable, proliferate and even enhance the expression of their original phenotype (22, 23, 34-36). Collagen is further known as the main matrix component

in cardiovascular tissues and with its special architecture crucial for biomechanical function (24, 37).

Using species of *S. aureus, S. sanguis* and *S. epidermidis* that are known pathogens causing endocarditis, we verify, that endothelial challenge with *S. aureus* and to a certain extent also with *S. sanguis* and *S. epidermidis* is sufficient to trigger diverse EC proinflammatory responses that promote TF-dependent coagulation, cytokine and chemokine production and monocyte adhesion that result in monocyte mediated amplification of TF-dependent coagulation. The bacterial strain and species specific probabilities to induce endothelial adherence and activation in vitro on tissue culture plates, as shown in the present study, are in essence confirmatory to earlier observations (4, 6, 9). Further detailed comparative analyses of our data, however, reveal additional interesting findings when ECs are seeded on the two gel matrices. The current values on EC infection obtained with *S. aureus* on fibrin and collagen gel show a 3 to 4 fold increased probability of infection compared to those found on tissue culture plates in this study and as reported by us earlier (9, 38), suggesting that the gel matrices provide adhesive cofactors for optimal bacterial adhesion to ECs. Different cell profiling on the gel matrices might contribute to increased adhesive capability. In addition, bacterial adhesion was found also moderately elevated for *S. sanguis* and *S. epidermidis*, when ECs were seeded on the two gels. Looking at the adhesion capacity to the gel matrices itself, we verified, that especially *S. aureus* is capable to bind effectively to fibrin ($p < 0.01$) and collagen gel compared to tissue culture plates. Since *S. aureus* surface molecules provide binding sites for collagen and fibrinogen (39-42), it is tempting to suggest that this observation could be explained by bacterial binding to the matrix rather than to endothelial adhesion. However, microscopic evaluation of the infected ECs verified an intact monolayer and showed that adherent bacteria were widely spread over the EC monolayer and not concentrated at the outer cell border, excluding that the high adhesion capacity is explained by adhesion to the matrix rather than to the endothelial cells seeded on the gel matrices. Interestingly, bacterial adhesion was equally observed on the fibrin and collagen matrix.

The novelty and major focus of our present study, however, was to critically evaluate the potential of bacterial induced EC activation upon cellular contact in the absence or presence of adhering monocytes with ECs seeded on the gel matrices. Earlier

studies, including our own investigations, reveal that *S. aureus* adhesion subsequently induces endothelial activation (7, 9, 43, 44). As a result these ECs acquire a proinflammatory and procoagulant phenotype, as shown by enhanced cell surface expressions of ICAM-1 and VCAM-1, secretion of IL-6, IL-8 and MCP-1 as well as monocyte adhesion and, as discussed in more detail below, an enhanced TF-dependent procoagulant activity.

Despite higher infection levels observed on the gel matrices compared to the tissue culture plates, *S. aureus* was equally effective in inducing EC adhesion molecule expression. This can be concluded by similar results evaluating activation levels of ECs cultured on all matrices. Moderate endothelial ICAM-1 and VCAM-1 production was, however, also observed upon infection with *S. sanguis* and *S. epidermidis* with similar values reached on all matrices. Apparently bacterial contact to the surface of intact ECs is sufficient to signal endothelial IL-8 and MCP-1 production.

From our further investigations we conclude that a bacteria-EC interaction mediated preferentially by *S. aureus*, but also by *S. sanguis* and *S. epidermidis* is a decisive determinant in the amplification of procoagulant activity in a setting in which monocytes are present. The importance of circulating monocytes settling on a *S. aureus*-infected endothelial or valvular surface for the maintenance of TFA and formation of fibrin clots has been assigned by us previously (4, 6, 8). Adding to this, the current data reveal that the monocyte mediated enhancement of TF-dependent coagulation in *S. aureus* infected ECs on tissue culture plates is not at all seen when ECs are cultured on fibrin and collagen gel.

This novel finding was rather unexpected. Importantly, this observation is not explainable in light of the results on monocyte adhesion to ECs since monocyte adhesion was found even higher when ECs are cultured on the two gel matrices. This is paralleled by a similar endothelial ICAM-1 and VCAM-1 expression on all matrices which enable monocytes to adhere. Lower levels of endothelial MCP-1 production on the gel matrices might play a role in monocyte chemotaxis but are unlikely to account for a decreased monocyte mediated generation of TFA.

Upregulation of TFA in cocultures of monocytes and *S. sanguis* and *S. epidermidis* infected ECs, which is in accordance with previous findings (9), was not observed on

fibrin and collagen gel but to moderate degree upon infection with *S. epidermidis* on tissue culture plates (6).

It is known from previous studies that direct contact of monocytes to endothelial cells is essential to augment the enhancement in TFA (6). In addition, results obtained in a three dimensional model show that direct EC-monocyte contact is required to elucidate full amplification of inflammation and coagulation activity (45, 46). Known that an intact EC monolayer on the surface of the gels was demonstrated by histological staining in our study we postulate that monocyte-endothelial contact is present when ECs are cultured on a three dimensional fibrin or collagen gel matrix.

The lack of monocyte enhanced TFA in ECs seeded on the gel matrices may, in essence, relate to down regulation of TFA by activation of inhibiting pathways. Previous studies on matrix adherent monocytes without ECs being cocultured reveal that monocytes are indeed capable to induce inhibition of TFA. In detail, fibronectin-adherent monocytes were found to express increased TF as well as marked increased amounts of TFPI which were efficient to reduce TFA (47). In contrast, non-adherent, endotoxin stimulated monocytes in the same study expressed significant amounts of TF but no TFPI (47). In addition, production of the anti-inflammatory cytokine IL-10 was found to play an important role in controlling TFA in monocytes adherent to a bacteria-infected fibrin matrix (48). In further different experimental settings, TFA downregulation via protein C mediated IL-10 production or IL-4 was shown (49, 50).

Therefore, cell-matrix interactions are likely to induce pathways which enable monocytes to down regulate TFA. If in our experimental setting, when ECs are cultured on fibrin and collagen gel, adherent monocytes are as well capable to produce TFPI and / or IL-10 which could explain the downregulation of TFA, is planed to be investigated in a following study.

Distinct mechanisms which enable monocytes to demonstrate TF regulative phenotypes are not known yet, but recent investigations of gene expression and cell proteomic changes in monocytes cultured on matrices support the thesis that the basic matrix contribute to the cell phenotype and functional determination (51, 52). Furthermore, not the underlying matrix might be essential but also the extracellular matrix which is produced by endothelial cells in culture seems to be important in this model (53, 54).

Together these studies may support the concept that in the phase of *staphylococcal* valve colonization, when monocytes augment coagulation and fibrin depositions, monocyte recruitment with direct cell to cell contact is ensured and induction of regulatory pathways favour control of thrombin generation. This is expected to be concomitantly influenced by applying mechanical stimulation and shear stress, which awaits further investigation.

Collectively, our findings give further insight in essential bacterial and cellular interactions in the early phases of bacterial endocarditis and show the relevance of a fibrin or collagen based matrix used in tissue engineering. The data reveal an equally high capacity of bacterial adhesion with ECs cultured on fibrin and collagen gel matrices which lead to subsequent endothelial inflammatory activation. Despite a marked monocyte adhesion we found no relevant enhancement of endothelial procoagulant response mediated by monocytes in ECs cultured on the gel matrices. Therefore, the fibrin and collagen matrix might inherit a potential positive effect in controlling thrombin generation and fibrin deposition at the infected site. This knowledge provides relevant targets to construct an optimal heart valve scaffold based on tissue engineering.

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CHAPTER VI

General Discussion

Infective endocarditis is defined as a bacterial infection of the endocardium, which covers the inner surface of the heart. Disease occurrence is severe and systemic complications are common, due to dissemination of bacteria via the bloodstream.
Despite progress in diagnostic and therapeutic strategies, IE remains associated with a high morbidity and mortality due to infection related factors and delay in diagnosis (1-3). IE is characterized by an inflammatory reaction at the infected endothelial site and especially by formation of a clot consisting of fibrin, platelets and the bacterial organism (4). This vegetation implicates severe dysfunction of the heart valve with release of disseminating bacteria and parts of the vegetation as septic emboli. In the early pathogenesis of endocarditis, the bacterial - endothelial interaction is crucial. Depending on their pathogenicity, bacteria attach either to intact endothelium or pre-damaged.

In the present thesis we identify Fn-binding surface molecules of *S. aureus* as critical molecules implicated in eliciting a proinflammatory and procoagulant phenotype of intact human ECs, which is essential in the initial phase of endovascular infection (5-8). Using surrogate non-invasive *L. lactis* recombinants, that express *staphylococcal* FnBPA, FnBPB or ClfA molecules, we found that the sole interaction of FnBPA, and to a certain extent also that of FnBPB, but not ClfA molecules with ECs is sufficient to trigger diverse EC proinflammatory responses that promote TF-dependent coagulation, cytokine and chemokine production and monocyte adhesion resulting in monocyte-mediated enhancement of TF-dependent coagulation.
In a further part of the thesis we focused on the contribution of single or combined FnBPA subdomains (A, B, C or D) to bacterial-endothelial interaction. *L. lactis* strains expressing single different FnBPA (sub)domains or combinations thereof were used to show that domains mediating Fn-binding, in contrast to others, were necessary and sufficient to provoke the typical EC responses.
In the final part of the thesis, it is illustrated that an underlying matrix used in heart valve tissue-engineering has an impact on the endothelial phenotype when ECs cultured on matrices are infected with *S. aureus*, *S. sanguis* and *S. epidermidis*. Data obtained upon *S. aureus* infection revealed a high capability to infect ECs and subsequent endothelial proinflammatory response. Despite this sufficient bacterial adherence and subsequent proinflammatory endothelial activation, monocyte derived

TFA was found to be downregulated in ECs cultured on fibrin and collagen gel compared to tissue culture plates. Therefore, we found evidence to support our research suggestion that underlying matrices do influence the EC phenotype in the experimental setting of essential steps in the early stage of endovascular infections, such as IE.

S. aureus inherits a high propensity to colonize endovascular tissues, which is facilitated by a variety of distinct bacterial surface proteins, collectively called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (9, 10).

Our data obtained with surrogate non-invasive *L. lactis* recombinants that express *staphylococcal* FnBPs confirm that FnBPA and to a lesser degree FnBPB surface molecules of *S. aureus* are involved in endothelial adhesion and invasion (9, 11-14). Since values achieved with FnBPA-positive *lactococci* resemble the data found with invasive *S. aureus* strains in earlier studies (7, 15), our results suggest that FnBPA and to a lesser degree FnBPB are effective to induce optimal bacterial adhesion to ECs without any adhesive cofactors needed. In contrast, ClfA inherited a low ability to mediate bacterial adhesion in vitro.

However, the novel finding of the study is that FnBPA and FnBPB surface expression is also required to induce an endothelial proinflammatory and procoagulant phenotype shown by enhanced cell surface expressions of ICAM-1 and VCAM-1, secretion of IL-6 and IL-8, but not IL-10, as well as monocyte adhesion and, as discussed in more detail below, an enhanced TF-dependent procoagulant activity. FnBPA- and FnBPB-expressing *lactococci* were found to be equally effective in inducing EC activation, although FnBPA-positive strains inherited a slightly higher capacity to confer EC infection.

FnBPA plays also a decisive role in the amplification of procoagulant activity in a setting in which monocytes are present. TF-dependent coagulation is significantly enhanced by monocytes adherent to ECs infected with FnBPA-positive strains in our study. Based on previous investigations, which identified the importance of circulating monocytes adhering to *S. aureus*-infected endothelium for the maintenance of TFA

and formation of fibrin clots, we state that the presence of FnBPA surface molecules is sufficient to induce the coagulation cascade in this setting (6, 8, 16).
Cellular adhesion as well as the generation of high levels of TFA in these coculture experiments are not explained by an interaction between integrin receptors on monocytes with their respective counterparts ICAM-1 and VCAM-1 on the infected ECs, but most likely involves other adhesive molecules presented by monocytes, infected ECs and/or their membrane-bound bacteria. This is concluded from the experiments where antibodies that block CD18 and CD49d mediated monocyte adhesion were used without showing any effect. A minor role of endogenous IL-1 and TNF-α is suggested from inhibiting experiments (8, 17).
Our findings parallel experiments done with an invasive *S. aureus* strain, suggesting, that mechanisms seen previously in *S. aureus* infection are resembled by infection using the FnBPA-positive mutant strain (8).

Interestingly, ClfA-expressing *lactococci* did not induce EC activation in vitro. Apparently, the interaction between ClfA-positive *lactococci* and ECs is not sufficient to signal EC activation. These findings go along with previous investigations where it was shown that such interaction also did not result in endothelial internalization of the ClfA-expressing *lactococci* (13, 18). Our data are in contrast with in vivo observations, identifying Fg-binding as a critical pre-requisite for IE initiation (12, 19) whereas in vitro EC activation seems to be solely Fn-mediated.

The FnBPA surface molecule combines both: Fg- and Fn-binding. The molecular structure of FnBPA reveals different domains mediating these properties as shown in figure 4. Several studies have identified the fibronectin binding part in the C- terminal part as well as the AB spanning region (20-23). The AB spanning region is characterized by overlapping of Fn and Fg binding capacities.
Based on the knowledge that FnBPA mediates endothelial adhesion and activation, as described in the first part of this thesis, we assigned the contribution of single or combined FnBPA (sub)domains (A, B, C or D) to bacterial-endothelial interactions. Using a large library of *L. lactis* expressing single different FnBPA (sub)domains or combinations thereof, we demonstrated that the expression of at least one Fn-binding module is necessary and sufficient to preserve its maximum potential to

infect human ECs and launch the typical EC proinflammatory and procoagulant responses.

Figure 4 summarizes the distinct domains which are known to facilitate Fn-binding and in addition, as proved in our experiments, facilitate endothelial adhesion and activation.

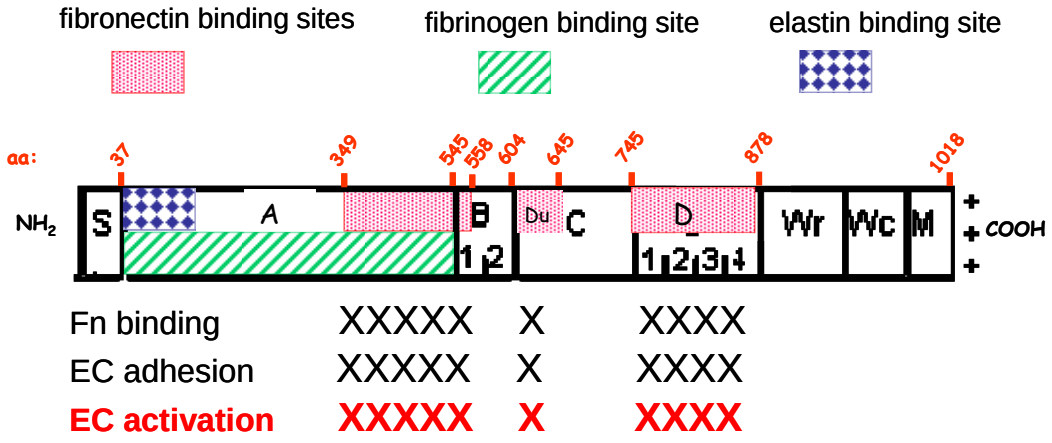

Figure 4: *Staphylococcal* FnBpA (sub)domains contribution to endothelial response
Subdomains named A, B, C, D and Du. S is the leader sequence, Wr and Wc are wall anchoring components, M a membrane component. Fn = fibronectin, EC = endothelial cell.

In detail, our experiments support the importance of these Fn-binding domains, both for binding and subsequent infection of cultured human ECs. Comparative data analysis showed that the capacity of the recombinant *lactococci* to bind Fn correlates with the magnitude of EC infection and also activation. The investigated *L. lactis* CD recombinant strain effectively binds Fn and is capable to induce EC adhesion, which was quite similar to the pattern observed with *L. lactis* expressing full length FnBPA (this thesis), or invasive *S. aureus* strains (5, 7, 15, 24). Moreover, these findings parallel competition experiments, in which recombinant CD fragments fully inhibit *S. aureus*-EC interactions (25).

Moreover, our current data reveal that EC infection and activation by recombinant *lactococci* is observed as long as the recombinant strains express an Fn-binding module. Residues containing 432-559 of the AB spanning region $A4^{+16}$, including

parts for Fg- and Fn-binding, were identified to mediate EC adhesion and activation in this thesis. These results confirm and extend the findings of another study that FnBPA fragment $A4^{+16}$ emerges as a critical region that harbours the EC adhesive and invasive ability of the FnBPA molecule (19). The FnBPA fragment $A4^{+16}$ was found to be the minimal required region for endothelial invasion in vitro and concomitant valve infection in animals (19).

Resuming this and earlier discussed results in animal experiments, the overall understanding suggests that Fg-binding is central in valve colonization and Fn-binding in endothelial activation (12, 19). Together, both pathways orchestrate disease severity.

FnBPA domains mediating Fg-binding alone were not able to induce endothelial adhesion and activation in our in vitro studies. But this thesis supports the conclusion that expression of at least one single Fn-binding region of FnBPA is sufficient to confer bacterial adhesion to human ECs in vitro without any additional co-factors needed.

As mentioned before, our results concerning the involvement of Fn-binding properties contrast with data reached in animal studies. Therefore, we are aware that interpretation of our results is limited to the fact that all experiments are done under static conditions in vitro. Especially the potential synergistic role of FnBPA and ClfA needs further detailed evaluation (12). As an advantage, in vivo investigations allow to study complex, interacting mechanisms. Thus, co-acting factors are excluded in our in vitro system giving the possibility to focus on specific questions.

A recent genetic study on carriage isolates of *S. aureus* demonstrated an apparently paradox finding. Carriage isolates of *S. aureus* which were even least adherent to Fn and Fg kept their ability to cause infection in vivo (26). Hence, differential gene expression, host factors and gene redundancy are discussed, which illustrates the complexity in vivo. In order to develop potential effective anti-adhesive treatment strategies, which need to target each of the various relevant Fn- and Fg-binding domains, knowledge of potential synergistic interaction is mandatory. Further experiments need to address co-acting abilities of the different *staphylococcal* binding molecules, e.g. FnBP and ClfA.

Together, the first part of this thesis supports the hypothesis that FnBPA mediated Fn-binding is of major importance in mediating initial *staphylococcal* colonization to the undamaged valve and concomitant activation of ECs in vitro. In the later phase of valvular infection, when fibrin-rich lesions are formed, FnBPA and ClfA may play complementary roles.

To address the question, if a fibrin or collagen matrix has an impact on bacterial-endothelial interaction, in vitro experiments were transferred to a setting where ECs are cultured on the mentioned matrices. Using species of *S. aureus, S. sanguis* and *S. epidermidis* that are known pathogens causing endocarditis (27, 28), we verify that endothelial challenge with *S. aureus,* and to a certain extent also with *S. sanguis* and *S. epidermidis,* is sufficient to trigger diverse EC proinflammatory responses that promote TF-dependent coagulation, cytokine and chemokine production and monocyte adhesion that results in potential monocyte-mediated amplification of TF-dependent coagulation.

Figure 5 summarizes *S. aureus* mediated bacterial adhesion and subsequent endothelial responses and indicates upcoming questions, as discussed below.

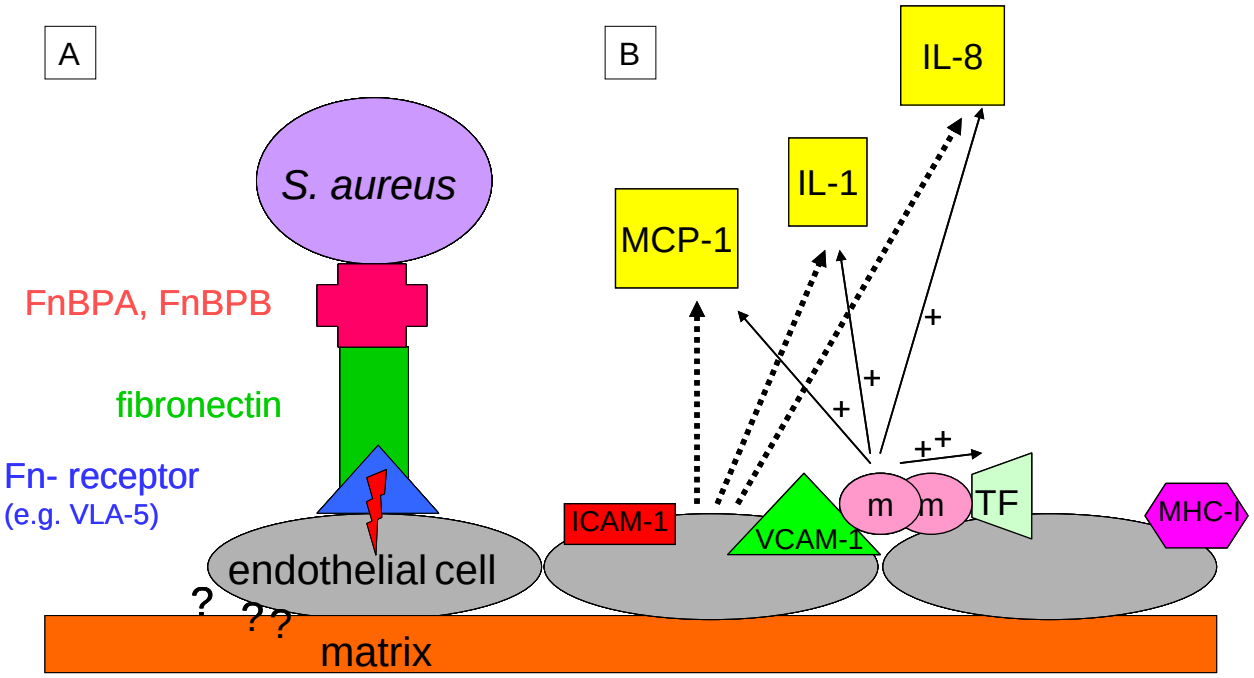

Figure 5: Bacterial-endothelial interaction and endothelial response in *S. aureus* infection

A) mechanisms of *S. aureus* adhesion to ECs, B) endothelial responses induced upon infection
m = monocytes, Fn = fibronectin

Since results reveal an increased endothelial infection upon *S. aureus* stimulation in ECs cultured on the gel matrices compared to ECs seeded on tissue culture plates, we assume that the gel matrices provide additional factors to facilitate bacterial adhesion. Despite different levels of *S. aureus* adhesion, the subsequently achieved inflammatory response measured by adhesion molecule expression was equally increased on the gel matrices and on tissue culture plates. This parallels the observations in the first part of our studies showing that the degree of inflammation is not strictly correlated to the amount of adherent bacteria, but fully upregulated when an adequate trigger is present. Compared to earlier investigations, data achieved on the two gels as well as on tissue culture plates resemble previous findings, which demonstrated a marked increase in adhesion molecule expression facilitating monocyte recruitment (8). Proinflammatory cytokines (MCP-1, IL-6, IL-8) produced by ECs and monocytes enhanced the inflammation (6, 29-31). Interestingly, *S. aureus* stimulated production of IL-8 and MCP-1 shows slightly higher levels after seeding ECs on fibrin gel vs. collagen gel.

Since fibrin and collagen are known players in different inflammatory and procoagulant pathways, especially known as a potent trigger of thrombosis, our interest was focused on the activation of the coagulation. As known, the effective activation of the coagulation system via TF leads to formation of a vegetation (thrombus) enveloping the bacteria to escape host defense mechanisms.

Upon contact with *S. aureus,* increased adhesion of monocytes resulted in a synergistic enhancement of TF-dependent coagulation on tissue culture plates. In contrast, TFA remained low in ECs seeded on the two gel matrices. This finding is rather unexpected since it is not explainable in light of the number of monocytes adherent to ECs (6, 8). Monocyte adhesion was equally present on all matrices. A downregulation of TFA by the onset of anticoagulant pathways might account for the low values found under these circumstances. This hypothesis is supported by a study performed on Fn-adherent monocytes, which reveals that these monocytes expressed increased TF as well as tissue factor pathway inhibitor (TFPI) which was efficient to reduce TFA (32). In turn, non-adherent, endotoxin stimulated monocytes expressed significant amounts of TF but no TFPI (32). In addition, IL-10 was found to

control monocyte TFA when monocytes are adherent to a bacteria-infected fibrin matrix (17). Fibrin adherent monocytes produced high levels of TFA, IL-1 and TNF-α, In turn, TNF-α induced secretion of the anti-inflammatory cytokine IL-10. IL-10 decreased TFA, which was confirmed by neutralisation of endogenous IL-10 preventing the downregulation of monocyte TFA (17). Going along with these findings, TFA downregulation via protein C mediated IL-10 production or IL-4 was shown in different experimental settings (33, 34). Based on these studies, we assume that onset of different TF inhibiting pathways via TFPI or anti-inflammatory cytokines might also be actively involved in reduction of TFA in our experimental setting. Evoking pathways facilitating monocytes to downregulate TFA interferes with ongoing fibrin formation and might have a positive effect on disease progression. Mechanisms regulating TFA in a setting where endothelial cells are involved and seeded on the matrices awaits further investigations.

S. sanguis and *S. epidermidis* inherit low abilities to infect ECs in vitro, which was confirmed in this thesis (7). But, these microorganisms reveal a synergistic enhancement of endothelial inflammatory response and TFA when monocytes are present.

Together, these findings lead to the conclusion that the fibrin and collagen gel matrix equally facilitate efficient bacterial adhesion launching endothelial proinflammatory response. The matrices might induce mechanisms to regulate procoagulant activity as suggested from a low monocyte mediated TFA. Results provide evidence that matrices contribute to EC phenotype expression influencing inflammation, tissue damage and fibrin deposition at the infected endovascular site.

Endothelium is exposed to shear stress in vivo. Shear stress was found to alternate mechanisms of *S. aureus* adherence mediated by the gene loci agr en sar (35). Investigations on FnBP induced *S. aureus* adhesion to resting ECs identified a preventive effect of shear stress (36). Future studies should evaluate the impact of shear stress in FnBPA mediated endothelial proinflammatory and procoagulant response as well as in matrix mediated endothelial phenotype expression.

Targeting these subjects of interest should clarify our insights in Fn-mediated *S. aureus*-endothelial cell interaction in accordance to more relevant in vivo like conditions.

SUMMARY

Infective endocarditis (IE) still remains a clinical challenge in diagnosis and is associated with severe complications. Results of this thesis give further insight in the understanding of the EC activation process induced by *S. aureus*, one of the most important players causing acute valvular disease.

Fibronectin binding proteins of *S. aureus* were verified to mediate bacterial adhesion to ECs. We first described the role of FnBPs in inducing a variety of proinflammatory endothelial responses, resulting in leukocyte accumulation, cell damage and fibrin deposition. Using recombinant lactococci expressing *staphylococcal* surface molecules FnBPA or FnBPB, we found a very efficient inoculum-dependent pattern of EC infection in these strains. This coincided with EC activation, as we found a marked increase in surface expression of ICAM-1 and VCAM-1 as well as secretion of IL-8 and IL-6. The FnBPA-induced surface expression of ICAM-1 and VCAM-1 was similar to that found after stimulation with IL-1, a potent inducer of endothelial pro-inflammatory responses.
FnBPA-positive *L. lactis* also induced a prominent tissue factor-dependent endothelial coagulation response by elevated levels of TF-antigen and factor Xa activity. Furthermore, FnBPA mediated monocyte adhesion, which coincided with a significant increase in the amount of FXa formed. The ClfA-positive *L. lactis* strain showed a very low probability to infect ECs, which did not lead to EC activation.
From these results we conclude that *S. aureus* FnBPs, but not ClfA, confer invasiveness and pathogenicity to non-pathogenic *L. lactis* organisms, indicating that bacterium-EC interactions mediated by these adhesins are sufficient to evoke inflammation as well as procoagulant activity at infected endovascular sites.

Further investigations addressed the contribution of the single or combined FnBPA subdomains (A, B, C or D) to the bacterial-endothelial interaction. Using *L. lactis*, expressing single FnBPA (sub)domains or combinations thereof, we demonstrated that domains which mediate Fn-binding were necessary and sufficient to provoke the typical EC responses.

FnBPA subdomains CD (aa 604-877) or $A4^{+16}$ (aa 432-559) are involved in bacterial adhesion to ECs, which correlates with the capacity of these fragments to elicit a marked increase in EC surface expression of both ICAM-1 and VCAM-1 and secretion of the IL-8 chemokine and finally to induce a tissue factor-dependent endothelial coagulation response. In addition, our current data reveal that that only one single Fn binding modules is sufficient for maximal stimulation of endothelial proinflammatory and procoagulant responses.

In summary, this study gives further insight in the role *S. aureus* FnBPA plays in facilitating EC activation processes. Results identified the essential role of the tandemly repeated Fn-binding modules, expressed either alone or in combination, to both infect and activate ECs.

In the final part of the thesis, our findings are transferred to an endocarditis model, which applies tissue engineering. Novel therapeutic strategies aim to minimize associated risk factors in valve replacement and to increase biocompatibility, by constructing autologous matrix-based tissue engineered heart valves.

The interaction of these matrices with bacteria is not known yet, but the potential role of these newly constructed heart valves, and especially their matrix, in the pathogenesis of endovascular infection seems important. Therefore, we compare the influence of a fibrin and collagen gel matrix on the bacterial-endothelial interaction, upon contact with *S. aureus*, *S. epidermidis* and *S. sanguis.*

Results established a high capability of *S. aureus* to infect endothelial cells seeded on a fibrin and collagen matrix. In contrast, *S. aureus* bacteria adhered to the ECs seeded on the tissue culture plate showed significantly lower values. Infection of ECs with *S. sanguis* and *S. epidermidis* showed a similar, limited infection rate on these three surfaces.

The bacterial adhesion capacity was similar on the two gel matrices and resulted in endothelial proinflammatory and procoagulant activation. We found an important endothelial activation with markedly expressed ICAM-1 and VCAM-1 values after *S. aureus* infection, compared to a low increase of endothelial activation after stimulation with *S. sanguis* and *S. epidermidis*.

Experimental results reveal no difference between the fibrin and collagen matrix but compared to results acquired on tissue culture plates a decreased monocyte

mediated procoagulant activity was observed despite unchanged monocyte adhesion. Enhanced anticoagulant pathways, such as production of tissue factor pathway inhibitor (TFPI) or IL-10, might be involved.

These investigations provide evidence for the importance of different matrices as inducers of pathways to evoke inflammation, tissue damage and fibrin deposition at infected endovascular sites.

Collectively, this thesis gives further insight in the essential bacterial and cellular interactions in the early phases of the pathogenesis of *S. aureus* endocarditis. To develop anti-adhesive treatment strategies, the knowledge that FnBPA is identified as a central surface molecule and that at least one Fn-binding domain is necessary and sufficient to infect human ECs and, concomitantly, to launch a proinflammatory and procoagulant endothelial phenotype, is fundamental.

SAMENVATTING

Infectieuze endocarditis (IE) gaat gepaard met ernstige complicaties en blijft nog steeds een klinische en diagnostische uitdaging. Resultaten voorgesteld in deze thesis beschrijven het endotheliale activatieproces, dat door *S. aureus* geïnitieerd wordt, één van de belangrijkste spelers in het veroorzaken van acute hartklepdeficiëntie..

Wij hebben nagegaan in welke mate fibronectine-bindende eiwitten van *S. aureus* verantwoordelijk zijn voor de adhesie van bacteriën aan endotheelcellen (EC). Vooreerst is de rol van FnBPs beschreven in het veroorzaken van een reeks pro-inflammatoire endotheliale reacties, dewelke resulteren in leukocytaccumulatie op endotheel, celbeschadiging en fibrinevorming. Door gebruik te maken van recombinante *lactococci*, die de membranaire staphylococcus molecules FnBPA en FnBPB tot expressie brengen, hebben we een zeer efficiënt inoculum-afhankelijk EC-infectiepatroon kunnen induceren in de resulterende stammen. Dit proces ging samen met EC-activatie, gemeten aan de hand van een opmerkelijke verhoging van de membraanexpressie van ICAM-1 en VCAM-1, alsook van de secretie van IL-8 en IL-6. De FnBPA-geïnduceerde membraanexpressie van ICAM-1 en VCAM-1 was vergelijkbaar met deze bekomen na stimulatie door IL-1, een krachtige inductor van endotheliale proinflammatoire reacties.

FnBPA-positieve *L. lactis* induceerde eveneens een prominente weefselfactor-afhankelijke endotheelcel-gemedieerde stollingsreactie, d.m.v. verhoogde TF-antigeenspiegels en factor Xa activering. Daarenboven veroorzaakte FnBPA monocyt-adhesie, die samenviel met een significante toename van de hoeveelheid gevormd FXa.

De ClfA-positieve *L.lactis* stam vertoonde een lage probabiliteit van endotheelcelinfectie, zonder aanleiding te geven tot endotheelcelactivatie. Uit deze bevindingen konden we besluiten dat *S. aureus* FnBP's, doch niet ClfA, in staat zijn om invasiviteit en pathogeniciteit te verlenen aan niet-pathogene *L. lactis* micro-organismen, hetgeen er op wijst dat bacterie-endotheelcelinteracties - bewerkstelligd door deze adhesie-molecules - voldoende zijn om inflammatie alsook procoagulante activiteit uit te lokken in geïnfecteerde endotheellocaties.

Verder onderzoek was gericht op de bijdrage van enkelvoudige of gecombineerde FnBPA subdomeinen (A, B, C of D) op de bacterie-endothelelcelinteractie. Door gebruik te maken van een grote bibliotheek van enkelvoudige *L. lactis*-geëxprimerende FnBPA (sub)domeinen, al dan in combinatie, hebben we aangetoond dat domeinen met Fn-bindende activiteit nodig, doch voldoende waren om typische EC-reacties uit te lokken.

FnBPA subdomeinen CD (aa 604-77) of $A4^{+16}$ (aa 432-559) zijn betrokken in bacteriële adhesie aan ECs, hetgeen correleert met de mogelijkheid van deze fragmenten om een uitgesproken stijging te bewerksteligen in EC-membraaanexpressie van ICAM-1 en VCAM-1 en van de secretie van het chemokine IL-8, zowel als van een opgereguleerde weefselfactor-afhankelijke endotheelcel-gemedieerde stollingsinductie. Daarenboven tonen onze data dat één enkele Fn-bindende module volstaat voor maximale stimulatie van endotheliale en procoagulante responsen.

Samengevat leveren deze bevindingen bijkomende inzichten in de rol die *S. aureus* FnBPA speelt in het vergemakkelijken van EC activatieprocessen. Onze resultaten belichtten de rol van tandem-gerepeteerde Fn-bindende modules, die geëxprimeerd kunnen worden, ofwel alleen of in combinatie, tijdens infectie en activatie van ECs.

In het laatste deel van de thesis werden onze bevindingen doorgetrokken naar een endocarditis-model, met een invalshoek van weefselmodellering. Nieuwe therapeutische strategieën hebben immers als doel om de - aan klepvervanging geassocieerde - risicofactoren te minimaliseren en om de bio-compatibiliteit te verbeteren, dit via de constructie van autologe matrix-gebaseerde weefselengineering van hartkleppen. De interactie tussen deze matrices en bacteriën is tot op heden niet gekend, maar de potentiële rol van deze nieuw-gebouwde hartkleppen - en vooral hun matrix – in het uitlokken van endovasculaire infecties -, lijkt belangrijk. Daarom hebben we de invloed van fibrine en collageen-gel matrix vergeleken op de bacterie-endothelcel interactie na contact met *S. aureus*, *S. epidermidis* en *Streptococcus sanguis*.

De resultaten lieten zien dat S. aureus in hoge mate in staat is om endotheelcellen te infecteren, wanneer deze gekweekt worden op fibrine of collageen. Daartegenover staat dat *S. aureus* adhesie aan ECs significant lager uitvalt, wanneer endotheelcellen uitgeplaat werden, rechtstreeks op niet-gecoate weefselcultuurplaten. Infectie van ECs met *S. sanguis* en *S. epidermidis* was eerder beperkt en vertoonde geen verschil voor ECs op de drie oppervlakken.
De bacteriële adhesie was vergelijkbaar voor beide gel-matrices en resulteerde in endotheliale proinflammatoire en procoagulante activering. We vonden een belangrijke EC-activering met markante expressie van ICAM-1 en VCAM-1, na *S. aureus* infectie, in vergelijking met de lage toename van EC-activering, na stimulatie door *S. sanguis* en *S. epidermidis* .

De onderzoeksresultaten toonden geen verschil tussen fibrine- en collageengel, doch vergeleken met resultaten bekomen voor endotheelcellen, gekweekt op niet-gecoate cultuurplaten, werd een verlaagde monocyt-gemedieerde procoagulante activiteit waargenomen, ondanks een onveranderde monocytadhesie. Opregulering van anticoagulante cascades, zoals de productie van TFPI (Tissue Factor Pathway Inhibitor) of IL-10 zijn eveneens mogelijk.
Dit onderzoek wijst dus op het belang van het type matrix als inductor van pathways, die leiden tot inflammatie, weefselschade en fibrine-afzetting, ter hoogte van geïnfecteerde endovasculaire lesies.

Samengevat, verhoogt deze thesis onze inzichten in essentiële aspecten van bacteriële en cellulaire interacties in de vroege pathogenese van *S. aureus* geïnduceerde endocarditis. Voor de ontwikkeling van anti-adhesieve behandelings-strategieën, zijn de identificatie van FnBPA als een centrale membraanmolecule en de bevinding dat ten minste één Fn-bindend domein nodig is, doch tevens volstaat om endotheelcellen te infecteren en, terzelfdertijd, proinflammatoire en procoagulante fenotypes uit te lokken, fundamenteel.

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