Inhibition of major integrin αVβ3 reduces Staphylococcus aureus attachment to sheared human endothelial cells.

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ABSTRACT (250 words)

Background. Vascular endothelial dysfunction with associated oedema and organ failure is one of the hallmarks of sepsis. While a large number of microorganisms can cause sepsis, *Staphylococcus aureus* is one of the primary etiological agents. Currently there are no approved specific treatments for sepsis and therefore the initial management bundle focuses on cardiorespiratory resuscitation and mitigation against the immediate threat of uncontrolled infection. The continuous emergence of antibiotic resistant strains of bacteria urges the development of new therapeutic approaches for this disease. *Objective.* The objective of this study was to identify the molecular mechanisms leading to endothelial dysfunction as a result of *Staphylococcus aureus* binding. ***Methods.*** *Stahpylococcus aureus* Newman and clumping factor A-deficient binding to endothelium were measured *in vitro* and in the mesenteric circulation of C57Bl/6 mice. The effect of the $\alpha V\beta 3$ blocker, cilengitide, on bacterial binding, endothelial VE-cadherin expression, apoptosis, proliferation and permeability were assessed. ***Results.*** Here we show that the major *Staphylococcus aureus* cell wall protein clumping factor A binds to endothelial cell integrin $\alpha V\beta 3$ in the presence of fibrinogen. This interaction results in disturbances in barrier function mediated by VE-cadherin in endothelial cell monolayers and ultimately cell death by apoptosis. Using a low concentration of cilengitide, ClfA binding to $\alpha V\beta 3$ was significantly inhibited both *in vitro* and *in vivo*. Moreover, preventing *Staphylococcus aureus* from attaching to $\alpha V\beta 3$ resulted in a significant reduction in endothelial dysfunction following infection. ***Conclusion.*** Inhibition of *Staphylococcus aureus* ClfA binding to endothelial cell $\alpha V\beta 3$ using cilengitide prevents endothelial dysfunction.

ESSENTIALS

- *Staphylococcus aureus* (*S. aureus*) binds and impairs function of vascular endothelial cells (EC)
- We investigated the molecular signals triggered by *S. aureus* adhesion to endothelial cells
- Inhibition of the EC integrin αvβ3 reduces *S. aureus* binding and rescues EC function
- αvβ3 blockade represents an attractive target to treat *S. aureus* bloodborne infections

INTRODUCTION

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection[1]. There is an estimated 20 million new cases of sepsis worldwide per year with a mortality rate of up to 50%[2]. The vascular endothelium is a significant target of sepsis-induced events and endothelial pertubation underlies systemic injury in sepsis[3]. For example, bacteria binding to endothelial cells results in activation and granule mobilization. This leads to von Willebrand factor deposition on the surface of the endothelial cells which contributes to rapid platelet translocation and thrombus formation[4]. A concomitant decrease of anticoagulation factors, with a reduction of thrombomodulin at the surface of the endothelial cell and a reduction of circulating levels of protein C leads to clot formation and triggers disseminated intravascular coagulation[5]. A breakdown of the endothelial barrier results in fluid leakage into the extravascular space leading to life threatening oedema in septic patients[6]. The inflammatory response also plays a key role in the sepsis phenotype and an excessive or sustained inflammatory response contributes to the tissue damage and death[7]. At present there is no effective specific anti-sepsis treatment. Besides the administration of intravenous fluids and vasopressors required to stabilize the patients, the infection is treated with aggressive intravenous combination antimicrobial therapy, frequently with meagre success[8],[9].

Recent evidence involving 14,000 ICU patients in 75 countries suggest that S*taphylococcus aureus* is one of the most frequently occurring underlying causes of sepsis and causes perturbation when it binds to the endothelium[10]. The success of *S. aureus* as an opportunistic pathogen in the cardiovascular system is due in part to

its expression of a wide array of Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMM's)[11]. Using these MSCRAMM's, *S. aureus* can attach either directly or indirectly to host cells to initiate infection. *S. aureus* clumping factor A (ClfA) is a major MSCRAMM and has already been shown to play a key role in bloodstream infection by binding to αIIbβ3 on platelets and inducing rapid thrombus formation under physiological conditions *in vitro*. Inhibition of αIIbβ3 or using a strain deficient in expression of ClfA, prevents thrombus formation[12]. The integrin αIIbβ3 is platelet specific and not expressed on endothelial cells however, another β3 integrin, αVβ3, is expressed on endothelial cells and interestingly has been shown to be upregulated in sepsis patients[13].

Using an ex vivo dynamic model of human endothelial cells, we identified a critical interaction between *S. aureus* ClfA and αVβ3 that results in endothelial cell apoptosis and loss of barrier integrity (increased permeability). Furthermore, we identified a compound that inhibits *S. aureus* from binding to endothelial cells and in doing so prevents the signals that result in apoptosis and increased permeability. These results have important implications for the treatment and management of sepsis.

Materials and Methods

Materials

All reagents used in this study were sourced from Sigma (Wexford, Ireland) unless otherwise stated. Bacteria were a kind gift from Professor Timothy Foster (Trinity

College Dublin). Cilengitide was a kind gift from Professor Horst Kessler (Technical University of Munchen, Germany).

Blood collection and plasma preparation

Whole blood was collected from healthy donors and anticoagulated with hirudin (300U/ml). Plasma was obtained as described previously[14]. Approval for the collection of whole blood was obtained from the Ethics Committee in RCSI (REC 679b). Informed consent was provided in accordance with the Declaration of Helsinki.

Bacteria growth conditions

Bacteria strains used in this study were: *Staphylococcus aureus* Newman Wildtype NCTC 8178[15], *Staphylococcus aureus* ΔClfA DU5876 (clfA::Erm^R defective in clumping factor A) [16], *Staphylococcus aureus* ΔSpA DU5971 (spa::Ka^R; defective in Protein A) [17], *Lactococcus lactis* mock transfected NZ9800 (pKS80 empty vector) [18], *Lactococcus lactis* +ClfA NZ9800 (pNZ8037 ClfA ErmR) [19], *Lactococcus lactis* +ClfA PY NZ9800 (pNZ8037 ::clfa PY Cam^R , expressing ClfA where P336 and Y338 are replaced with serine and alanine respectively) [20], *Lactococcus lactis* +SpA NZ9800 (pKS80 spa) [21]. All strains were cultured anaerobically at 37°C overnight and prepared as described previously[12].

Cell culture conditions

In order to develop a dynamic ex vivo model that represents the physiological state of human blood vessels, primary derived Human Aortic Endothelial Cells were cultured and subjected to haemodynamic shear force in Endothelial Cell Growth

Media as previously described (Promocell, Germany)[22]. Unless otherwise stated, endothelial cell infection was preceded by incubation with 10ng/mL TNFα for 4hr at 37°C and 5% CO_2, followed by exposure to plasma, fibrinogen (4mg/ml) or IgG (1-8mg/mL) for 1hr. In some experiments cells were preincubated with the αVβ3 antagonist, cilengitide (0.05μM), for 1hr in between the TNFα exposure and the addition of plasma/fibrinogen.

Binding assays

Sheared endothelial cells in the presence of TNFα and plasma/fibrinogen or 40μg/mL of purified recombinant αVβ3 (R&D systems, UK) were immobilized onto microtitre plates, blocked with 1% BSA for 2hrs at 37ºC and incubated with $1x10^7$ SYBR green II stained bacteria at a MOI of 400 for a further hour at 37°C. In some experiments cilengitide was preincubated with the cells or purified protein for 1h prior to addition of the bacteria. Wells were finally washed gently to remove non-adhered bacteria. Fluorescence was read at 485/535 nm in a plate reader (1420 Victor V3, Perkin Elmer, Ireland) before (Reading1) and after (Reading2) the final wash. Number of adhered bacteria per well was computed as (Reading2/Reading1) x $1x10^7$.

αvβ3 analysis by flow cytometry

αVβ3 expression pre and post- activation with TNFα (10ng/ml for 4hrs) was measured by flow cytometry. Endothelial cells were incubated with anti-αVβ3 (LM609) or istoype control. Primary antibodies were incubated with the endothelial cells for 1hr at 37ºC followed by a FITC-labelled secondary antibody in the dark. After 20 min incubation the samples were analysed on a flow cytometer (Becton

Dickinson, Oxford, UK) on the Fl-1 channel. Data were analysed using CellQuest software (Becton Dickinson).

Dot blots and western blots

Whole cell dot blots were carried out as previously described [23]. Briefly, a 10μl spot of bacteria was placed on a nitrocellulose membrane, allowed to dry at room temperature, blocked with 5% dry skimmed milk and probed with anti-mouse ClfA (1:1000) or anti-mouse SpA (Sigma, Ireland, 1:1000) antibodies. For western blots endothelial cells were lysed in RIPA buffer and proteins separated on a 10% SDS-PAGE. Separated proteins were transferred onto nitrocellulose membranes, blocked for 1h with 5% milk and probed with mouse VE-cadherin primary antibody (Santa Cruz, 1:1000). Anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Biolegend, 1:5000) were used as the secondary antibodies. Proteins were detected using ECL chemiluminescence (Millipore, UK).

Ca^{2+} mobilization in endothelial cells following infection

Sheared endothelial cells were loaded with the Ca^{2+} sensitive dye Fluo-4 AM (5μM, Molecular Probes) for 20 minutes. Endothelial cells were then perfused with HEPES buffer containing 10ng/mL TNFα and 0.4mg/mL fibrinogen, and switched to the same buffer containing bacteria after 2 minutes. Samples were excited at 488nm and >500nm emission was measured at 2 minute intervals. Live images were acquired using an epifluorescence microscope (Zeiss AxioObserverZ1). Timecourse of endothelial cell intracellular Ca^{2+} levels were expressed as Fluo-4 fluorescence F/F_0 (F_0=initial fluorescence).

Immunofluorescence

Endothelial cells were stained with either VE-cadherin mouse monoclonal (1:50, InVitrogen), vWf rabbit polyclonal (1:50, BD Biosciences), and fluoresced using a secondary Alexa-fluor® 488 donkey-anti rabbit or donkey-anti mouse (1:200, Invitrogen). Coverslips were mounted on slides with fluorescent mounting medium containing DAPI for nuclei staining (ProLong, Invitrogen). Control experiments were performed incubating samples with primary or secondary antibody alone. Images were acquired using an inverted epifluorescence microscope. For extracellular vWF quantification, 10 images were captured per field (Metamorph), background was subtracted, nuclei counted and vWf attributable fluorescence measured (ImageJ). Extracellular vWf was computed as fluorescence intensity per cell, dividing vWf fluorescence by the number of nuclei present in each image.

Quantification of endothelial cell proliferation and apoptosis

Endothelial cell proliferation was determined by counting cells on a haemocytometer in a 1:1 dilution with Trypan Blue after a 24h period and comparing it to the seeding density. Apoptosis was determined by flow cytometry. Following the 24h infection, endothelial cells were trypsinized, washed and resuspended in ice cold PBS. The endothelial cells were pelleted in FACS tubes and re-suspended in the reagent mix of TACS™ Annexin V Kits (AMS Biotechnology, Oxford) according to the manufacturer instructions. Endothelial cells were incubated in the dark at room temperature for 15mins and analyzed on a flow cytometer (BD FACSCanto™ II Flow Cytometer).

Permeability assay

Endothelial barrier permeability was assessed as previously described[24]. Briefly, sheared endothelial cells were plated at a density $2x10^5$ cells/mL on the upper chamber of hanging inserts, (Millicel, Millipore, pore size 0.4μM) and endothelial cell media was added to the lower chamber. Following infection, FITC dextran (250μg/mL; 40kDa) was added to the endothelial cells in the upper chamber. Permeability was determined after 24hrs by measuring the amount of FITC dextran that permeated through the endothelial cells into the lower chamber using a fluorescent plate reader (1420 Victor V3, Perkin Elmer). Data is expressed as a percentage of 100% permeability.

Mesenteric Perfusion Model

S. aureus binding to mice mesenteric endothelium was measuring as previously described[4]. Briefly, six to eight week old C57Bl/6 mice were fasted and anaesthetized with ketamine/xylazine (125/12.5 mg/kg, intraperitoneal). The endothelium on the mesenteric circulation was exposed and activated with A23187 (10mM). Bacteria were labelled with 5(6)-carboxy-fluorescein N-hydroxysuccinimidyl ester and injected through the jugular catheter. Time-lapse images were acquired using an inverted fluorescence microscope. Fluorescent signal in the blood vessel corresponding to bound bacteria was quantified manually for each frame and the average computed as arbitrary fluorescence units. In some experiments 23.5ng/kg cilengitide was added to the animals for 5mins before addition of bacteria. Animal experiments were approved by the Ethical Committee of the University of Leuven.

Statistics

Data are presented as mean ± SEM. Experiments were carried out in triplicate with a minimum of three independent experiments. Statistical difference between groups was assessed by ANOVA with Tukey post-hoc test or t-test, as indicated. $P<0.05$ was considered significant.

RESULTS

Staphylococcus aureus binds to endothelial cells grown under haemodynamic shear force.

To examine the effect of shear stress on endothelial cell structure and *S. aureus* binding, monolayers of endothelial cells were grown under static conditions or exposed to fluid shear stress at 10dynes/cm^2 for 24hrs to mimic the conditions arterial cells experience *in vivo*. Images show that endothelial cells grown under static conditions exhibit a random “cobble stone” morphology and exhibit a distinct lack of immunostaining for adherens junction protein Vascular Endothelium (VE)-cadherin staining at the endothelial cell-cell border (Supplementary figure 1A). In contrast, endothelial cells sheared at 10dynes/cm^2 aligned in the direction of flow and there was clear staining of VE-cadherin at the plasma membrane of the endothelial cells at sites of cell-cell contact (Supplementary figure 1B). Consistent with this, statically grown endothelial cells expressed less VE-cadherin protein than sheared endothelial cells (Supplementary figure 1C). Formation of adherens junctions by VE-cadherin contributes to the functional barrier role of the endothelium [25], and as such sheared endothelial cells better represent the cellular morphology observed in the vasculature and constitute a more physiologically relevant model to study endothelial cell-bacteria interactions.

We observed no significant effect on *S. aureus* binding to sheared endothelial cells when either plasma proteins or TNFα were added separately. However, addition of human plasma and TNFα combined resulted in a significant increase in *S. aureus* binding to sheared endothelial cells (Figure 1). Interestingly, we also observed that *S. aureus* binding in the presence of plasma and TNFα is significantly higher for sheared endothelial cells than statically grown (Supplementary Figure 1D). Based on these observations, unless otherwise stated, all further experiments were performed on sheared endothelial cells in the presence of human plasma/fibrinogen and TNFα.

Staphylococcus aureus **ClfA mediates binding to sheared endothelial cells**

Major *S. aureus* cell wall proteins, Protein A (SpA) and ClfA have been previously identified as key players in the recognition of various host cells [12, 26]. Using isogenic mutants of these proteins we investigated their role binding to endothelial cells. A *S. aureus* mutant defective in expression of SpA (ΔSpA), failed to affect binding to sheared endothelial cells. In contrast, a *S. aureus* strain defective in expression of ClfA (ΔClfA) bound to a significantly lesser extent to endothelial cells (Figure 2A). Lack of expression of either SpA or ClfA on *S. aureus* was confirmed by dot blot (Figure 2C).

To confirm our finding, ClfA was expressed in the surrogate host *Lactococcus lactis*. We chose *L. lactis* as it naturally lacks the virulence factors present in *S. aureus*, in particular ClfA and SpA. Consistent with our previous findings, expression of SpA in *L. lactis* failed to increase binding to the endothelial cells above the mock transfected control, whereas expression of ClfA resulted in significant binding to endothelial cells

(Figure 2B). Over expression of SpA and ClfA in the surrogate host *L. lactis* was confirmed by dot blot (Figure 2D).

Fibrinogen acts as a bridge between ClfA and endothelial cells

S. aureus often binds a plasma protein to 'bridge' the bacteria host cells[27]. Previously we and others demonstrated that ClfA is capable of binding the plasma proteins IgG and fibrinogen both individually and simultaneously [12, 20]. To determine if *L. lactis* ClfA is binding these plasma proteins we added purified IgG or fibrinogen to endothelial cells, followed by incubation with *L. lactis* ClfA. Our results showed that IgG (up to 8mg/mL) failed to achieve significant levels of *L. lactis* ClfA attachment to endothelial cells. In contrast, purified fibrinogen (4mg/ml) restored the ability of *L. lactis* ClfA to bind to endothelial cells at levels similar to those observed in the presence of plasma (Figure 2E). These results are consistent with our observations with *S. aureus* where there is a significant reduction in binding to endothelial cells in the absence of fibrinogen (Supplementary figure 2). Deletion of the amino acids in ClfA (ClfA-PY) critical for binding fibrinogen [20], significantly reduced in its ability to bind to endothelial cells compared to *L. lactis* ClfA (Figure 2F).

***Staphylococcus aureus* ClfA mobilizes calcium and elicits deposit of von Willebrand factor onto the surface of endothelial cells.**

Calcium is an important second messenger and mobilization in endothelial cells results in Weibel-Palade body secretion[28]. Von Willebrand factor (vWf) is the primary constituent of Weibel-Palade bodies and once secreted attaches to the

surface of endothelial cells creating a binding site for *S. aureus* proteins SpA[29] and von Willebrand factor binding protein (vwbp) [4]. The nature of the signal that results in vWf secretion following S. aureus binding is currently unknown. We therefore measured endothelial cell intracellular Ca^{2+} following *L. lactis* ClfA infection by loading cells with Fluo-4-AM and surface vWf levels by immunofluorescence. Uninfected endothelial cells exhibited background levels of Fluo-4 fluorescence over a 12min period (Figure 3A&D). Addition of mock *L. lactis* exerted no significant effect on baseline Fluo-4 fluorescence (Figure 3B&D). Addition of *L. lactis* ClfA resulted in a significant transient increase in Fluo-4 fluorescence in endothelial cells (Figure 3C&D).

Neither the uninfected nor mock transfected *L. lactis* resulted in secretion of vWf onto the surface of the endothelial cells (Figure 3E&F). Addition of *L. lactis* ClfA to the endothelial cells elicited a significant increase in vWf on the surface of the cells (Figure 3G&H).

Staphylococcus aureus **ClfA binds αVβ3 integrin on endothelial cells both *in vitro* and *in vivo.***

A number of fibrinogen binding receptors have been previously described on endothelial cells including ICAM-1, α5β1 and αVβ3[30]. Preincubation of endothelial cells with monoclonal antibodies against ICAM-1 or α5β1 failed to have any effect on *L. lactis* ClfA binding to endothelial cells (Figure 4A). Preincubation of endothelial cells with αVβ3 antagonist, cilengitide (0.05μM) significantly reduced *L. lactis* ClfA from binding to endothelial cells compared to the vehicle control (Figure 4A). To exclude the possibility that cytotoxic effects of cilengitide were the underlying cause

of reduction in *L. lactis* binding to endothelial cells, we examined the effects of cilengitide on endothelial cells alone. No adverse effects on growth, cytotoxicity, apoptosis or VE-cadherin expression was detected on endothelial cells preincubated with cilengitide for 24hrs (Supplementary figure 3A-F). Consistent with these findings, cilengitide also significantly inhibits *S. aureus* from binding to endothelial cells (Figure 4B). Preincubation of cilengitide with purified αVβ3 in the presence of fibrinogen significantly inhibited *L. lactis* ClfA from binding (Figure 4C). Consistent with the finding that αVβ3 is up regulated in sepsis patients [13] we also found that αVβ3 expression is increased by 51% on our sheared endothelial cells following TNFα treatment (Table 1).

To validate our findings that ClfA binds to αVβ3 *in vivo* we used real-time videomicroscopy of the murine splanchnic veins and demonstrated rapid local accumulation of *S. aureus* on the vessel wall (Figure 5A&D). Innoculation of *S. aureus* ΔClfA resulted in a significant reduction in bacteria adhering to the vessel wall (Figure 5B&D). Furthermore, cilengitide (0.0005μM) substantially decreased *S. aureus* attachment to the vessel wall endothelium of the mice (Figure 5C&E).

Staphylococcus aureus **ClfA binding to αVβ3 inhibits proliferation and induces apoptosis in endothelial cells**

Uninfected sheared endothelial cells proliferated as expected over 24hrs however addition of *S. aureus* to endothelial cells significantly reduced the rate of proliferation (Figure 6A). Notably, addition of a *S. aureus* ΔClfA (Figure 6A) or addition of

cilengitide (Figure 6B) exhibited a significantly attenuated effect on endothelial cells proliferation compared to *S. aureus*.

Given our finding that the endothelial cell number decreased following 24hrs in the presence of *S. aureus*, we examined whether this was due to apoptosis. To determine this, Annexin V exposure on the cell surface, a hall mark of apoptotic cells, was assessed by flow cytometry. Uninfected endothelial cells have a low level of apoptosis, however upon addition of *S. aureus* to the endothelial cells, apoptosis was significantly increased. Addition of *S. aureus* ΔClfA caused a significantly lower extent of apoptosis in the endothelial cells (Figure 6C). Similarly preincubation of endothelial cells with cilengitide led to a significant reduction in *S. aureus* induced apoptosis (Figure 6D).

Staphylococcus aureus ClfA binding to αVβ3 induces increased vascular permeability

We hypothesize that *S. aureus* ClfA binding to αVβ3 generates a signal resulting in an increase in permeability a common feature in patients with sepsis. To test this we measured the paracellular permeation passage of FITC-dextran 40kDa across a confluent monolayer of endothelial cells in the presence and absence of *S. aureus*. Addition of *S. aureus* to the sheared endothelial cells led to a significant increase in barrier permeability (Figure 7A). Addition of *S. aureus* ΔClfA failed to increase permeability compared to wildtype *S. aureus*. Similarly, inhibition of αVβ3 with cilengitide attenuated the increase in permeability induced by wildtype *S. aureus*.

(Figure 7A). Consistent with these results, immunofluorescent staining of VE-cadherin on endothelial cells indicate tight barrier formation in uninfected samples (Figure 7B). However, upon infection with *S. aureus*, VE-cadherin expression in the cell membranes is reduced, suggesting cell-cell detachment (Figure 7C). Consistent with our previous data using the ΔClfA strain or treating the endothelial cells with cilengitide restores VE-cadherin expression and thus barrier integrity (Figure 7D and E, respectively).

DISCUSSION

In the present study, we used a dynamic model of endothelial infection that replicates endothelial conditions experienced during sepsis. Using sheared human endothelial cells we showed significant *S. aureus* binding in the presence of human plasma and low level of TNFα. Attachment was mediated by the *S. aureus* major cell wall protein, ClfA which in the presence of plasma fibrinogen bound to αVβ3 expressed on human endothelial cells. Binding resulted in calcium mobilization, granule exocytosis and vWf deposition on the surface of the endothelial cells. Within 24hrs of *S. aureus* attachment there was a significant loss of barrier integrity resulting in increased endothelial permeability. In parallel with elevated permeability following *S. aureus* infection of endothelial cells, we also observed impaired proliferation in conjunction with elevated apoptosis. Strikingly, the αVβ3 antagonist cilengitide, significantly reduced all these effects.

S. aureus ClfA is a major MSCRAMM expressed on the surface of all naturally occurring *S. aureus* strains and most critically uses fibrinogen to bridge to endothelial cells. While *S. aureus* expresses other fibrinogen binding proteins such as Clumping factor B (ClfB) and fibronectin binding protein A and B (FnbpA and FnbpB) we could not detect any involvement in their ability to bind to endothelial cells (data not shown). Although our experimental design allowed us to isolate the *S. aureus*-Endothelial cell interaction mechanism triggered by ClfA, the participation of other MSCRAMMs in clinical isolates of *S. aureus* cannot be ruled out. Indeed *S. aureus* ΔClfA exhibited a residual capacity of binding endothelial cells *in vitro* and *in vivo*, as well as in proliferation and apoptosis.

Previously, vWf has been shown to mediate *S. aureus* attachment to activated endothelium under flow conditions via vwbp[4]. In the current study we demonstrate that *S. aureus* ClfA binding to $\alpha v\beta 3$ induces calcium mobilization and exocytosis of Weibel-Palade bodies leading to deposition of vWf on the surface of endothelial cells. These results suggest that ClfA binding to $\alpha V\beta 3$ provides the signal that leads to vWf deposition on the surface of the endothelial cell and therefore provide a platform for the *S. aureus* vwbp to attach and anchor the bacteria to the vessel wall. Typically ultra large vWf multimers deposited on the surface of endothelial cells are cleaved by a disintegrin and metalloproteinase with a thrombospondin type I motif, member 13 (ADAMTS13) [31]. However patients with sepsis have an acquired deficiency of ADAMTS13 that leads to an inability to break down these ultra large vWf multimers[32] thus resulting in more *S. aureus* attachment and rapid progression of sepsis. Although the vWf has been reported to bind $\alpha V\beta 3$ under shear the contribution of this interaction in sepsis is still unclear[33].

During sepsis the vascular endothelial barrier breaks down which facilitates the passage of large molecules (such as albumin and plasma proteins) and leukocytes from the blood into the subendothelial compartment. This leads to life threatening oedema in the lungs, kidneys and brains of septic patients[34]. Using a dynamic ex vivo model that represents the physiological state of human blood vessels by forming tight junctions between the cells, we support this finding *in vitro* where *S. aureus* infection leads to a significant increase in endothelial permeability. Vascular endothelial (VE)-cadherin is a type I transmembrane protein and is an important adherens junction protein that plays a critical role in the maintenance and control of cell contacts that form the endothelial barrier. Significantly we demonstrate that addition of *S. aureus* to the sheared endothelial cells destabilizes the VE-cadherin interactions leading to an increase in permeability. Using a strain deficient in expression of ClfA or blocking $\alpha V\beta 3$ with cilengitide resulted in a significant reduction in endothelial cell permeability induced by *S. aureus* and a stabilization of the VE-cadherin contacts. We therefore suggest that preventing the ClfA $\alpha V\beta 3$ interaction with cilengitide arrests the signal that leads to apoptosis and subsequent reduction in VE-cadherin expression, thus reducing the possibility of an increase in vascular permeability. Interestingly, Alghisi et al., previously demonstrated that cilengitide binding to $\alpha V\beta 3$ on HUVEC resulted in down-regulation of VE-cadherin, thus contributing to increased vascular permeability[35]. We did not find this in our studies, however the concentration of cilengitide used in the Alghisi study was 200 times higher than what we used in our study (10μM versus 0.05μM, respectively).

Three integrins containing the αV subunit are expressed in endothelial cells: αVβ1, αVβ3 and αVβ5[36-38], of which only integrin αVβ3 has been described to bind fibrinogen with high affinity[39], and is thus the relevant integrin in fibrinogen-ClfA mediated *S. aureus* binding to endothelial cells. Cilengitide was originally developed for the treatment of glioblastomas and reached phase III clinical trials however treatment did not improve the overall survival of patients and the trials were suspended[40, 41]. The current study provides consistent evidence that cilengitide prevents ClfA from binding αVβ3 on the endothelium, impeding the activation of injurious pathways resulting in apoptosis and increased vascular permeability. We propose that Cilengitide could slow infection from progressing to multi-organ failure without compromising normal endothelial cell function. We therefore suggest that cilengitide represents a candidate drug for investigation of its potential therapeutic value, used in conjunction with antibiotics, to treat sepsis early in the infective process.

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Conflict-of-interest disclosure

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: PMC, SWK. Performed the experiments: CMD, CDG, RW, TMH, AML, JC. Data Analysis: CMD, CDG, RW, TMH, AML, JC, PV, PMC SWK. Manuscript preparation: CDG, PV, PMC, SWK.

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FIGURE LEGENDS

Figure 1. Sheared endothelial cells constitute a dynamic ex vivo model for the study of *S. aureus* binding. *S. aureus* adhesion to sheared endothelial cells, tested in the presence and absence of plasma and 10 ng/ml of TNFα. Equal amounts of endothelial cells were plated in microtitre plates. After blocking with 1%BSA, cells were infected with known amounts of SYBR Green I labelled *S. aureus* Newman (10^7) were added and incubated for 1 hr. Total fluorescence was measured on a plate reader at 485/535 nm. Unbound bacteria were removed by gently washing with PBS, and attached bacteria fluorescence was acquired. Results are expressed as number of bacteria attached per well. * $P<0.05$ vs. all other groups.

Figure 2. Clumping factor A binds fibrinogen to bridge *S. aureus* to endothelial cells. Binding of bacteria to sheared Endothelial cells (10 dyn/cm^2-24 hours) was measured at a MOI of 400 in the presence of plasma and 10ng/ml TNFα, as previously described. **(A)** Binding of *S. aureus* wild type (Newman), deficient in protein A (ΔSpA) or deficient in clumpling factor A (ΔClfA) to sheared Endothelial cells. **(B)** Binding of the surrogate host *Lactococcus* lactis, either mock transfected or expressing SpA or ClfA to sheared Endothelial cells..**(C)** The absence of the SpA and ClfA cell wall proteins was confirmed by dot blot. Blots are representative of three independent experiments. **(D)** Expression of ClfA and SpA on *L. lactis* was confirmed by dot immunoblots of whole bacteria cell lysates. Images are representative of three independent experiments. **(E)** Effect of the presence of plasma, IgG (1 mg/ml) or fibrinogen (4 mg/ml) on *L. lactis* ClfA binding to sheared

Endothelial cells. **(F)** Binding to sheared Endothelial cells of *L. lactis* expressing ClfA or ClfA PY in the presence of 4 mg/mL fibrinogen. N=3, **P*<0.05 vs. wildtype, ***P*<0.05 vs. all other groups, ANOVA.

Figure 3. ClfA induces intracellular Ca^{2+} increase and von Willebrand factor deposition the surface of endothelial cells. Endothelial cells were sheared for 24 hours, trypsinized and lawned in Ibidi microperfusion chambers. Cells were preloaded with Fluo-4 AM for intracellular Ca^{2+} detection by epifluorescence microscopy under flow (10 dyn/cm^2) at 37^0C with 40X oil immersion lens objective on a Zeiss AxioObserverZ1 epi-fluorescence microscope coupled to a CCD camera and equipped with mercury lamp and appropriate filters. Light intensity was adjusted to prevent signal saturation. After initial 2 minutes perfusion with HEPES solution containing 10ng/ml TNFα and 4 mg/ml fibrinogen, the perfusate was switched, in order to induce infection, to the same solution containing OD_{600} 0.6-0.7 *L. lactis* WT (n=8), *L. lactis* ClfA (n=6) or no bacteria (uninfected control, n=8). Samples were excited at 488 and >500nm emission was measured at regular time intervals. Representative "fire" pseudocolor images corresponding to **(A)** uninfected, **(B)** *L. lactis* WT infected and **(C)** *L. lactis* ClfA infected Endothelial cells, acquired 2 minutes after the infection. Scale bar represents 50μm. **(D)** Fluo-4 semiquantitative timecourse expressed as F/F_0 (F_0=at the time of infection), for uninfected (continuous line), *L. lactis* WT (dashed line) and *L. lactis* ClfA (dotted line) infected Endothelial cells. Arrow indicates the start of infection. For vWf measurements, sheared Endothelial cells (24 hours) were exposed to *L. lactis* WT or *L. lactis* ClfA in the presence of fibrinogen (4 mg/mL) and TNFα (10 ng/mL) for 60 minutes under 10 dyn/cm^2 shear. Uninfected cells were used as control. Same ImageJ brightness and

contrast settings were applied to all displayed images. Scale bar represents 50 μm. Representative images of surface vWf (yellow) detected by immunofluorescence of non-permeabilised cells (plan-apochromat 63x/1.40 oil immersion objective (Ex/Em 488nm/>505nm for vWf and 350/> 400nm for DAPI). corresponding to uninfected **(E)**, infected with *L. lactis* WT **(F)** and infected with *L. lactis* ClfA groups **(G)**. Nuclei were stained with DAPI (blue).

(H) Semiquantitative analysis of vWf levels in the surface of Endothelial cells, computed as fluorescence/cell (see methods) (n=3). **P*<0.05 vs. all other groups, ANOVA.

Figure 4. Fibrinogen bridges ClfA to endothelial cell αVβ3 integrin. (A) Sheared endothelial cells (10 dyn/cm^2-24 hours) in the presence of fibrinogen (4 mg/mL) and TNFα (10 ng/mL) were preincubated with either isotype/vehicle control, anti-ICAM1 (20 μg/ml), Anti-α5β1 (20 μg/ml) or cilengitide (0.05 μM) for 30 minutes prior to addition of *L. lactis* ClfA (n=3, *P<0.05 vs all other groups, ANOVA0). **(B)** Effect of 0.05 μM cilengitide on *S. aureus* binding to sheared endothelial cells (n=3, **P*<0.05, t-test). **(C)** Effect of cilengitide (0.05 μM) on *L. lactis* ClfA adhesion to immobilized purified αVβ3 integrin cilengitide (n=3, **P<0.001).

Figure 5. *S. aureus in vivo* adhesion to the endothelium is mediated by ClfA. *S. aureus* were injected into the right jugular vein of anesthetized mice, and their adhesion to the activated endothelium was measured by timelapse fluorescence recording in the mesenteric veins (one image per second). Representative images showing adhesion of fluorescent *S. aureus* WT to mesenteric veins **(A)**. Adhesion is diminished when ClfA is knocked out (*S. aureus* ΔClfA) **(B)** and is virtually abolished

by pre-administration of intravenous cilengitide (0.005μM) **(C)**. Quantitative bacteria adhesion computed as bacteria fluorescence average over 40 seconds (see methods). (D) Knocking out ClfA in *S. aureus* reduces attachment to endothelium, (n=10-17, **P*<0.05, t-test), (E) *S. aureus*-endothelium binding is significantly reduced by 0.005μM cilengitide and ΔClfA (n=11-17, ***P*<0.005, t-test).

Figure 6. Loss of Endothelial cells proliferation and induction of apoptosis induced by *S. aureus* is attenuated by deleting ClfA or blocking αVβ3 integrin with cilengitide. $1.5x10^5$ cells were seeded per well (time=0), and Endothelial cells proliferation was evaluated as the cell count after 24 hours (time=24). **(A)** Endothelial cells proliferation evaluated for uninfected cells (continuous line), infected with *S. aureus* Newman (dotted line) and *S. aureus* ΔClfA (dashed line) (n=3, * and ***P*<0.05 vs. all other groups, ANOVA). **(B)** Endothelial cells proliferation evaluated for uninfected cells (continuous line), infected with *S. aureus* Newman (dotted line) and with *S. aureus* Newman in the presence of αVβ3 integrin blocker cilengitide 0.05 μM (dashed line) (n=3, * and ***P*<0.05 vs. all other groups, ANOVA). For apoptosis assays endothelial cells were detached from plates by trypsinization and stained with Annexin-V antibody. Apoptosis levels were assessed by flow cytometry. **(C)** *S. aureus* Newman induced a significant increase in apoptosis, that was not observed with *S. aureus* ΔClfA (n=3, **P*<0.05 vs. all other groups). **(D)** αVβ3 integrin blocker cilengitide 0.05μM reduced apoptosis induced by *S. aureus* infection (n=3, * and ***P*<0.05 vs. all other groups, ANOVA).

Figure 7. Increase of endothelial permeability induced by *S. aureus* binding through ClfA is reduced by blocking αVβ3 integrin with a low concentration of cilengitide. (A) The barrier function of Endothelial cells monolayers was assessed by measuring the passage of FITC-dextran 40KDa added in the top compartment to the bottom compartment using transwell inserts. Endothelial cells were infected with *S. aureus* Newman or ΔClfA for 24 hours (MOI 400). After 24 hours, concentration of FITC-dextran on the bottom compartment was measured on a fluorescence plate reader. Values were calibrated between 100% permeability (absence of endothelial cells and bacteria) and 0% permeability (uninfected monolayer of sheared endothelial cells) (n=3, * and ***P*<0.05, ANOVA). In order to visualise the expression of VE-cadherin following infection endothelial cells were fixed, permeabilised and incubated with antibody against VE-cadherin (primary) and Alexa Fluor® 488-conjugated (secondary). Nuclei were stained with DAPI. Images were acquired with a fluorescence microscopy. Representative immunofluorescence images of three independent experiments showing **(B)** Uninfected endothelial cells, **(C)** *S. aureus* infected endothelial cells, **(D)** ΔClfA infected endothelial cells and **(E)** *S. aureus* infected endothelial cells in the presence of cilengitide.

TABLE 1. Integrin aVb3 is significantly increased on human aortic endothelial cells following infection with *Staphylococcus aureus*. Expression of αVβ3 was measured by flow cytometry in the presence and absence of TNFα (10ng/ml). Sheared endothelial cells were incubated with rabbit polyclonal anti-αVβ3 or isotype control. Primary antibodies were incubated with the endothelial cells for 1hr at 37ºC followed by a FITC labelled secondary antibody in the dark. αVβ3 expression was analysed on a FACS Calibur flow cytometer (Becton Dickinson, Oxford, UK) on the Fl-1 channel. P<0.05.

FIGURE LEGENDS (Supplementary)

Supplementary Figure 1.Development of a dynamic ex vivo model that represents the physiological state of human blood vessels. Confluent monolayers of HAoECs were fixed, permeabilized and incubated with antibody against VE-cadherin (primary) and Alexa Fluor® 488-conjugated (secondary). Nuclei were stained with DAPI. Images were acquired with a fluorescence microscopy. Immunofluorescence images are representative of three independent experiments. Statically grown cells display random orientation and patches with lack of VE-cadherin staining despite the presence of nuclei **(A)**; whereas for sheared cells, VE-cadherin and DAPI staining show alignment of cells and nuclei parallel to the direction of the flow **(B)**. VE-cadherin expression determined by western blot of protein homogenates from static and sheared HAoECs **(C).** *S. aureus* adhesion to static and sheared endothelial cells, tested in the presence of plasma and 10 ng/ml of TNFα. After priming with plasma and/or TNFα, equal amounts of endothelial cells were blocked with 1%BSA and subsequently infected with fluorescently SYBR Green I stained *S. aureus* Newman at a MOI of 400 for 1 hr. Total fluorescence was measured on a plate reader at 485/535 nm. Unbound bacteria were removed by gently washing with PBS, and attached bacteria fluorescence was acquired **(D)** (n=3, *P<0.01).

Supplementary Figure 2.Fibrinogen plays a critical role in the attachment of *S. aureus* to human aortic endothelial cells. The ability of *Staphylococcus aureus* to

bind to endothelial cells in the presence and absence of fibrinogen was tested. Equal amounts of endothelial cells were plated in 96 well microtitre plates in the presence of TNFα (10ng/ml). Fibrinogen (1mg/ml) or vehicle control (PBS) was added for 1 hr at 37ºC. After blocking with 1% BSA, endothelial cells were infected with SYBR Green I labelled *S. aureus* Newman ($1x10^7$) for 1 hr. Total fluorescence was measured on an plate reader at 485/535 nm. Unbound bacteria were removed by gently washing with PBS, and attached bacteria fluorescence was acquired. Results are expressed as number of bacteria attached per well. Black bars = *Staphylococcus aureus*, White bars = *Staphylococcus aureus* deficient in expression of ClfA. **P*<0.05 vs. all other groups using ANOVA.

Supplementary figure 3 Cilengitide alone does not induce any toxic effects on human aortic endothelial cells. Confluent monolayers of endothelial cells treated with 10 ng/ml TNFα for 4 hrs were exposed to a vehicle control **(A)** or 0.05μM cilengitide **(B)** for 24 hours to assess viability of cells. Cell metabolic activity was measured using a MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay **(C).** Apoptosis was measured using flow cytometric analysis of Annexin V staining **(D)**. Barrier integrity was measured by visualising fluorescently labelled tight junction protein, VE-cadherin in the presence and absence of cilengitide. Representative images of VE-cadherin (green) of control **(E)** and 24 hour 0.05 μM cilengitide treated **(F)** sheared endothelial cell monolayers.

Figure 1.

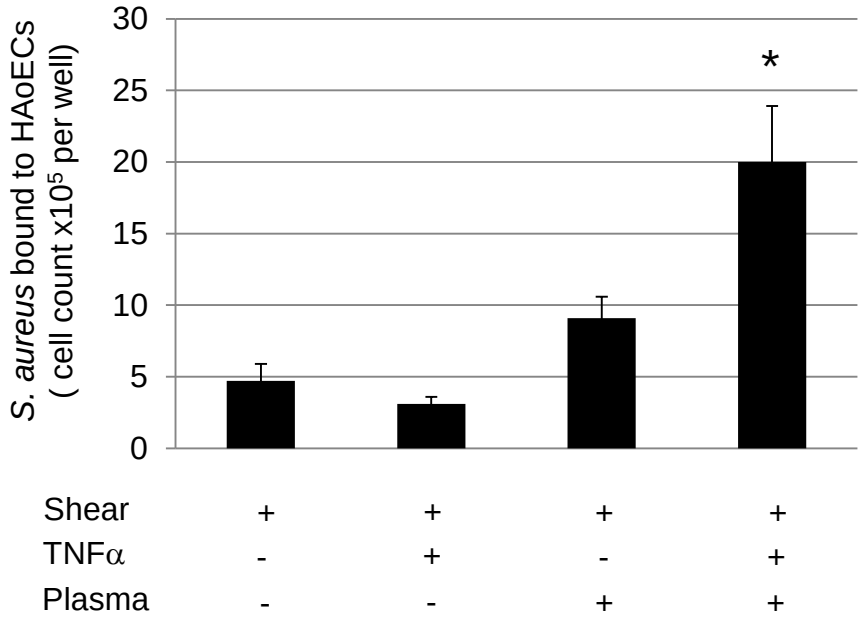

S. aureus bound to HAoECs
(cell count x10^5 per well)
30
25
20
15
10
5
0
*
Shear + + + +
TNFα - + - +
Plasma - - + +

Figure 2.

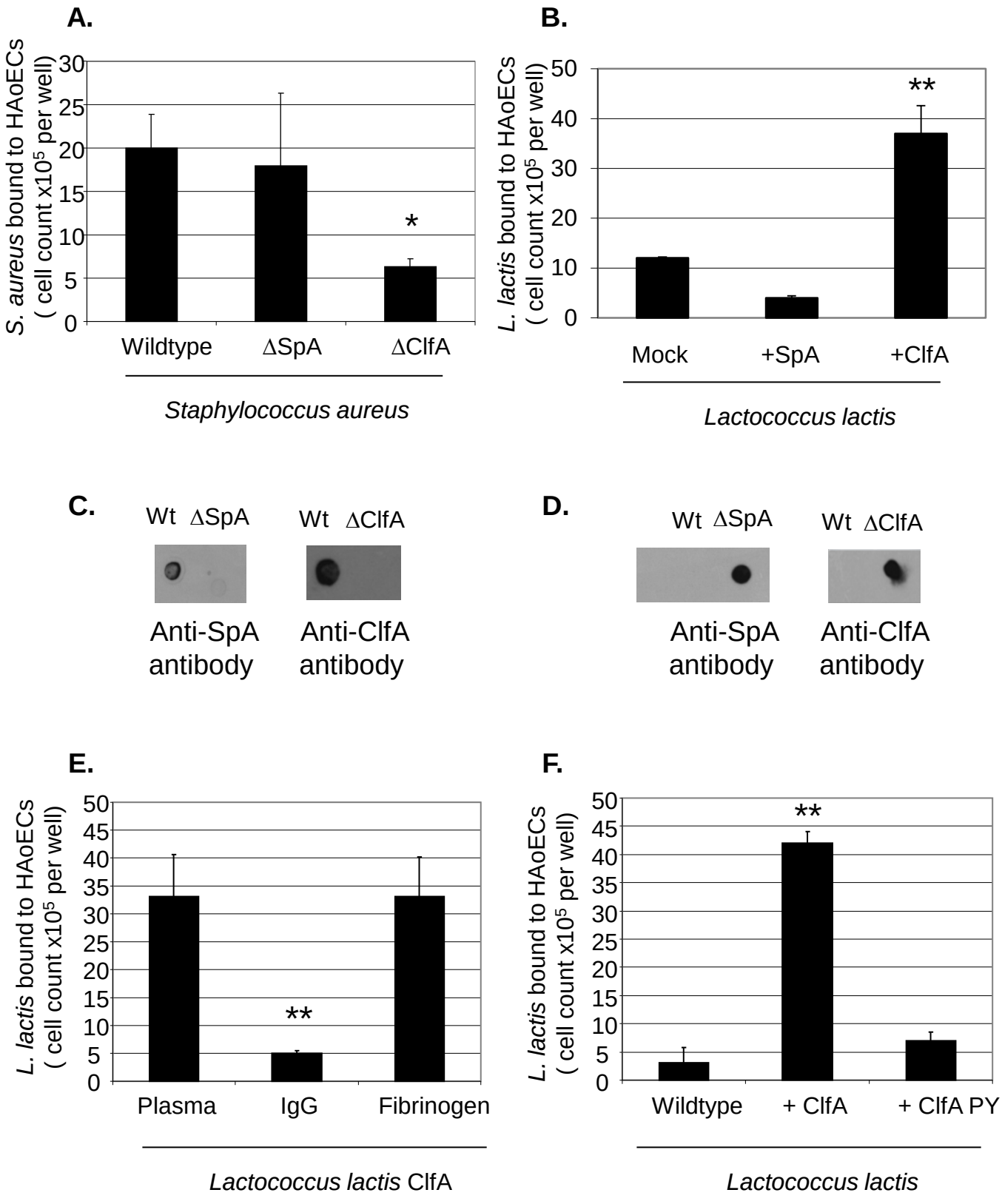
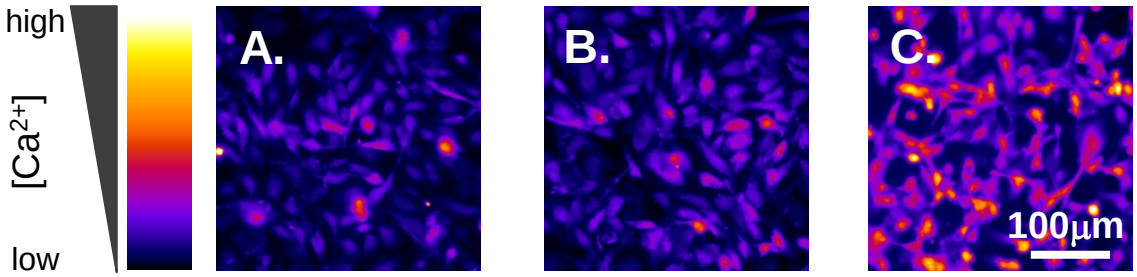
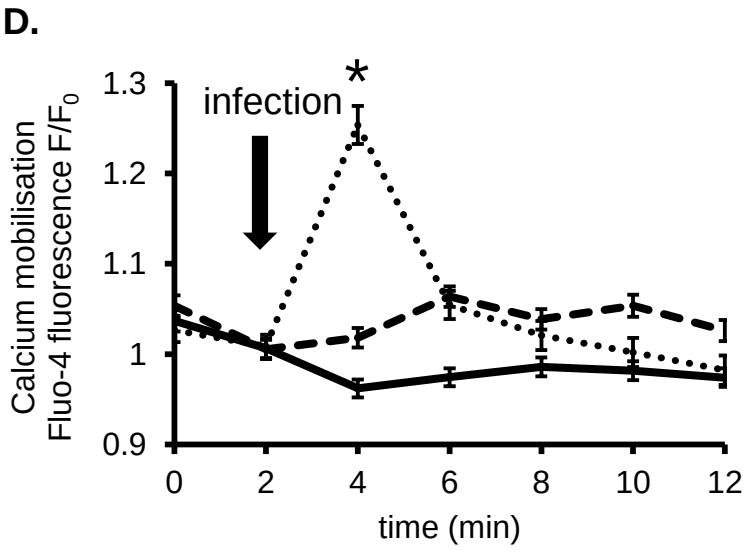
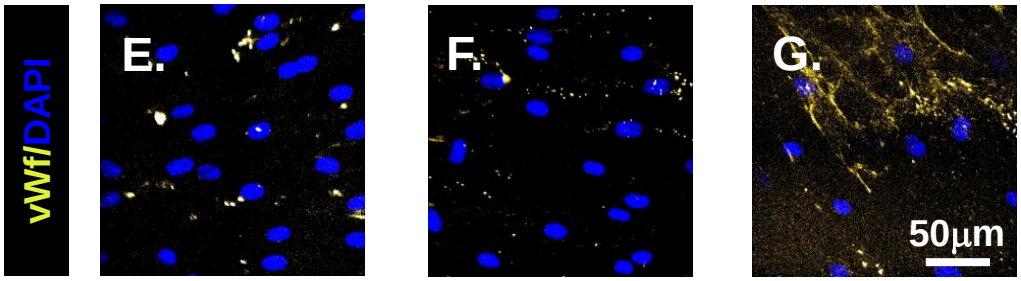

A.
S. aureus bound to HAoECs (cell count x10^5 per well)
30
25
20
15
10
5
0
*
Wildtype
ΔSpA
ΔClfA
Staphylococcus aureus
B.
L. lactis bound to HAoECs (cell count x10^5 per well)
50
40
30
20
10
0
**
Mock
+SpA
+ClfA
Lactococcus lactis
C.
Wt ΔSpA
Wt ΔClfA
Anti-SpA antibody
Anti-ClfA antibody
D.
Wt ΔSpA
Wt ΔClfA
Anti-SpA antibody
Anti-ClfA antibody
E.
L. lactis bound to HAoECs (cell count x10^5 per well)
50
45
40
35
30
25
20
15
10
5
0
**
Plasma
IgG
Fibrinogen
Lactococcus lactis ClfA
F.
L. lactis bound to HAoECs (cell count x10^5 per well)
50
45
40
35
30
25
20
15
10
5
0
**
Wildtype
+ ClfA
+ ClfA PY
Lactococcus lactis

Figure 3.


high
[Ca2+]
low
A.
B.
C.
100µm


D.
infection
*
Calcium mobilisation
Fluo-4 fluorescence F/F0
1.3
1.2
1.1
1
0.9
0
2
4
6
8
10
12
time (min)


vWf/DAPI
E.
F.
G.
50µm

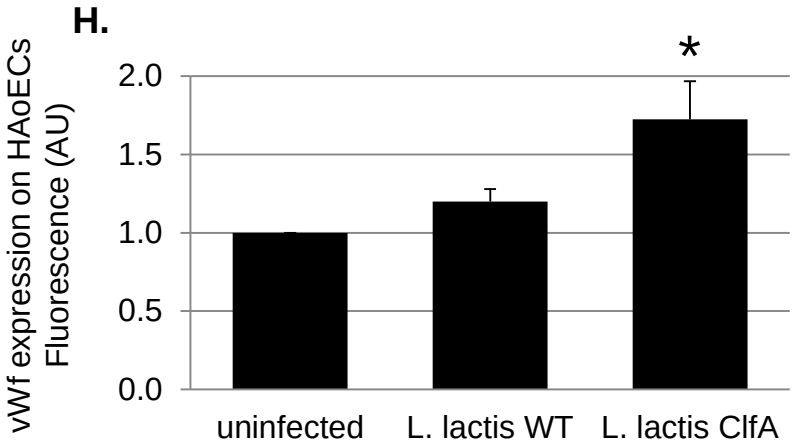
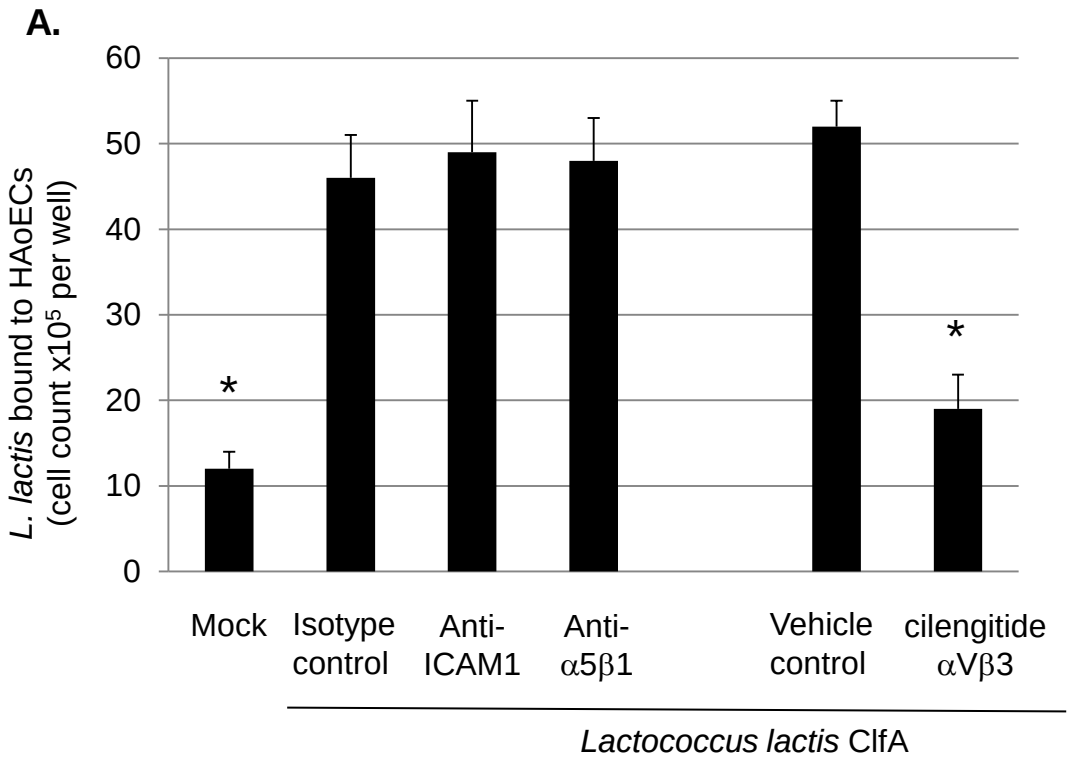
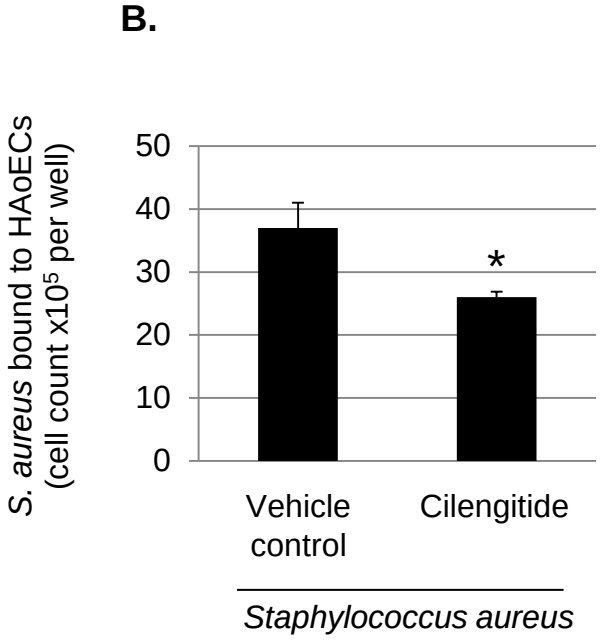

H.
*
vWf expression on HAoECs
Fluorescence (AU)
2.0
1.5
1.0
0.5
0.0
uninfected
L. lactis WT
L. lactis ClfA

Figure 4.


A.
L. lactis bound to HAoECs (cell count x10^5 per well)
60
50
40
30
20
10
0
*
*
Mock
Isotype control
Anti-ICAM1
Anti-α5β1
Vehicle control
cilengitide αVβ3
Lactococcus lactis ClfA


B.
S. aureus bound to HAoECs (cell count x10^5 per well)
50
40
30
20
10
0
*
Vehicle control
Cilengitide
Staphylococcus aureus

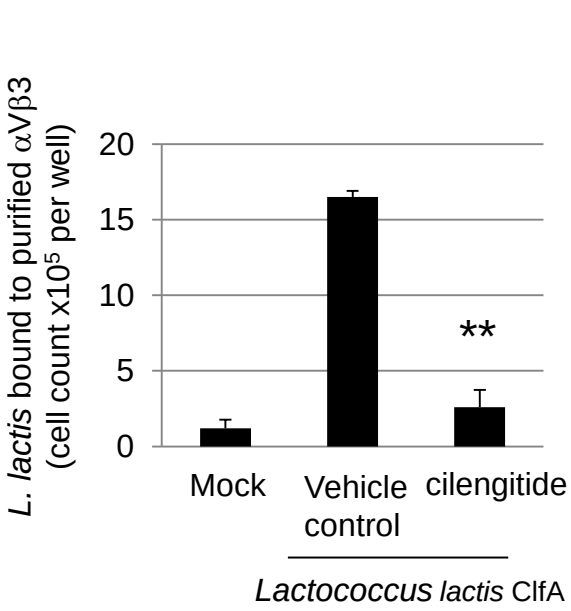

C.
L. lactis bound to purified αVβ3 (cell count x10^5 per well)
20
15
10
5
0
**
Mock
Vehicle control
cilengitide
Lactococcus lactis ClfA

Figure 5.

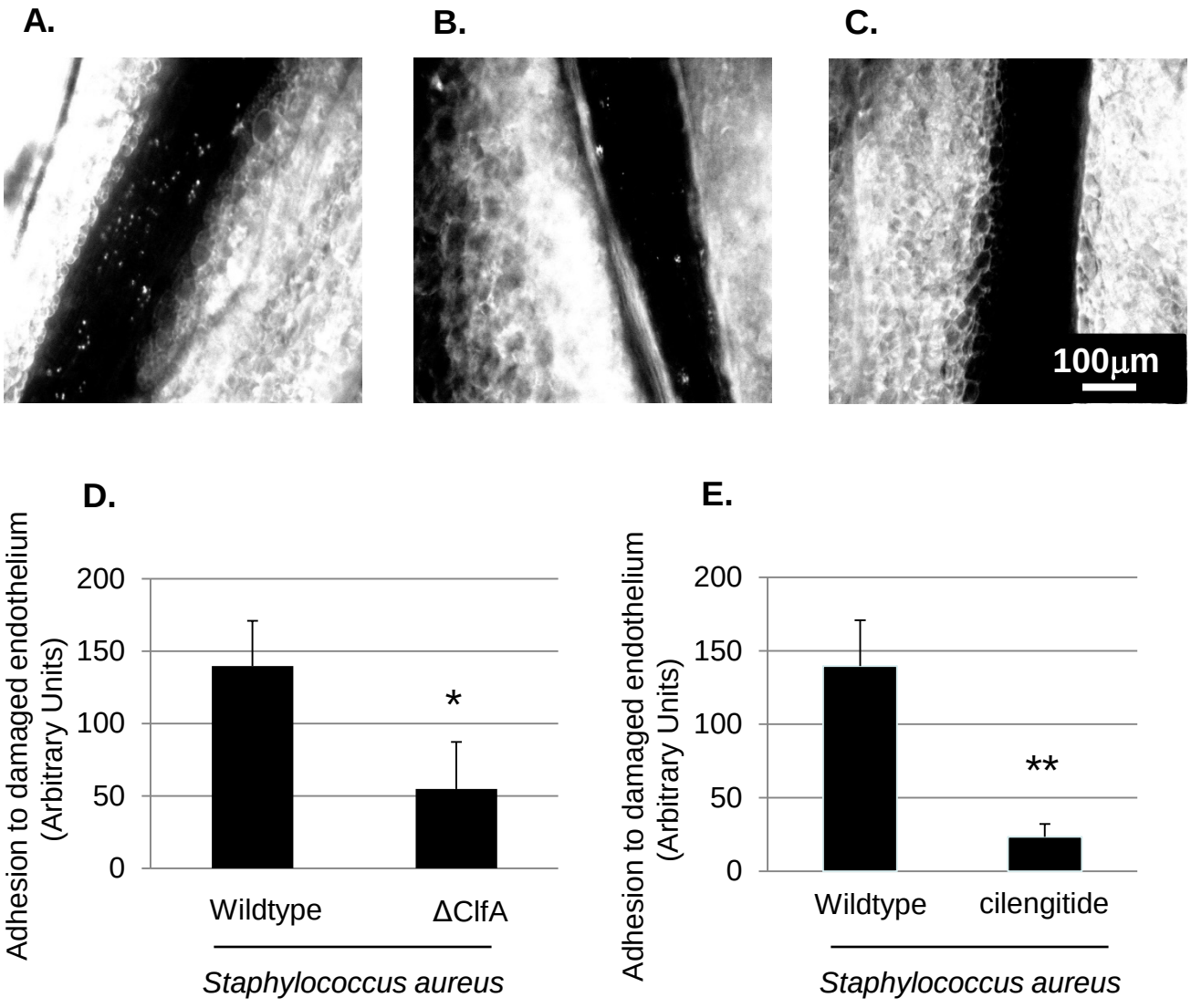

A.
B.
C.
100μm
D.
Adhesion to damaged endothelium (Arbitrary Units)
200
150
100
50
0
*
Wildtype
ΔClfA
Staphylococcus aureus
E.
Adhesion to damaged endothelium (Arbitrary Units)
200
150
100
50
0
**
Wildtype
cilengitide
Staphylococcus aureus

Figure 6.

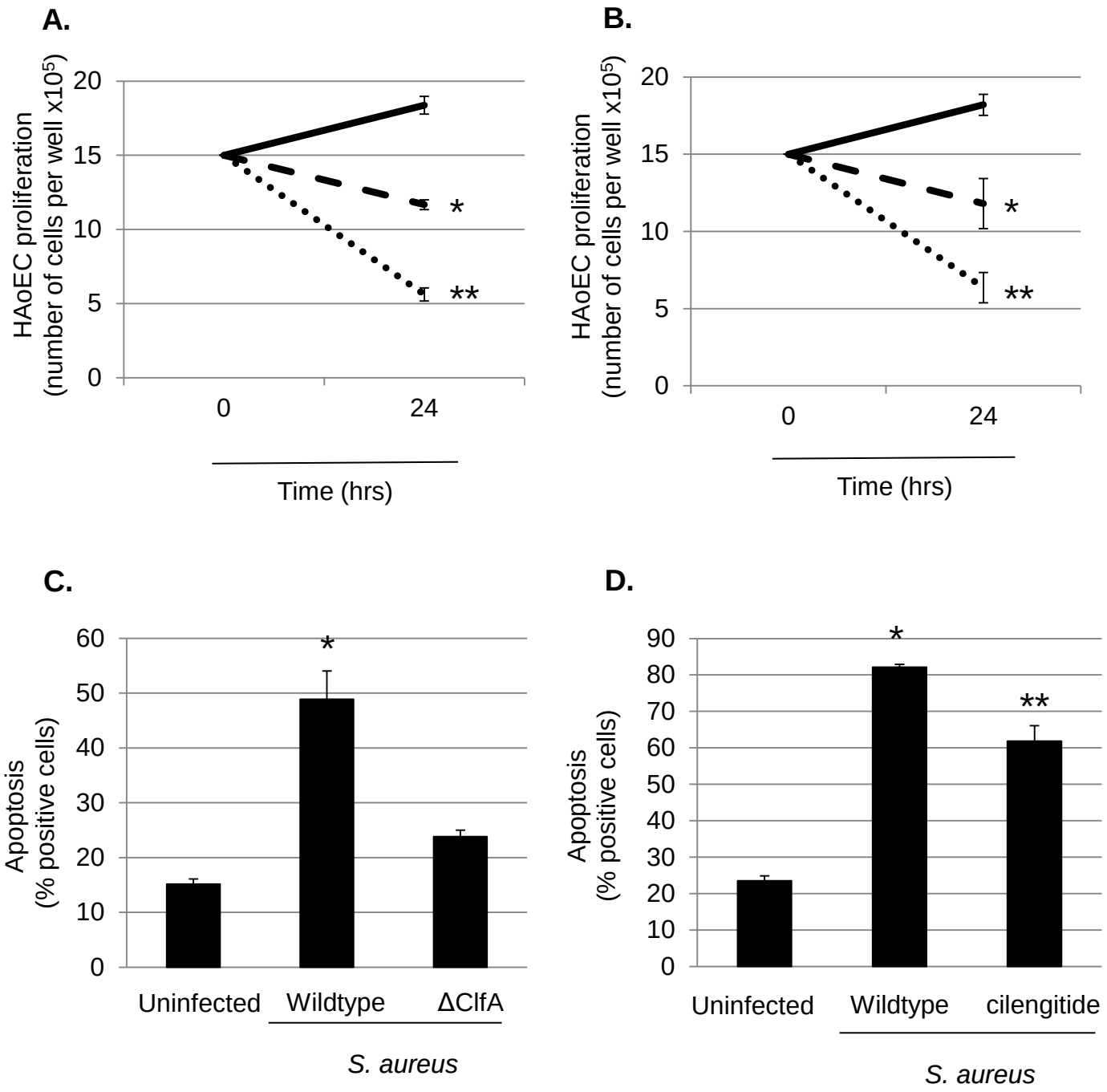
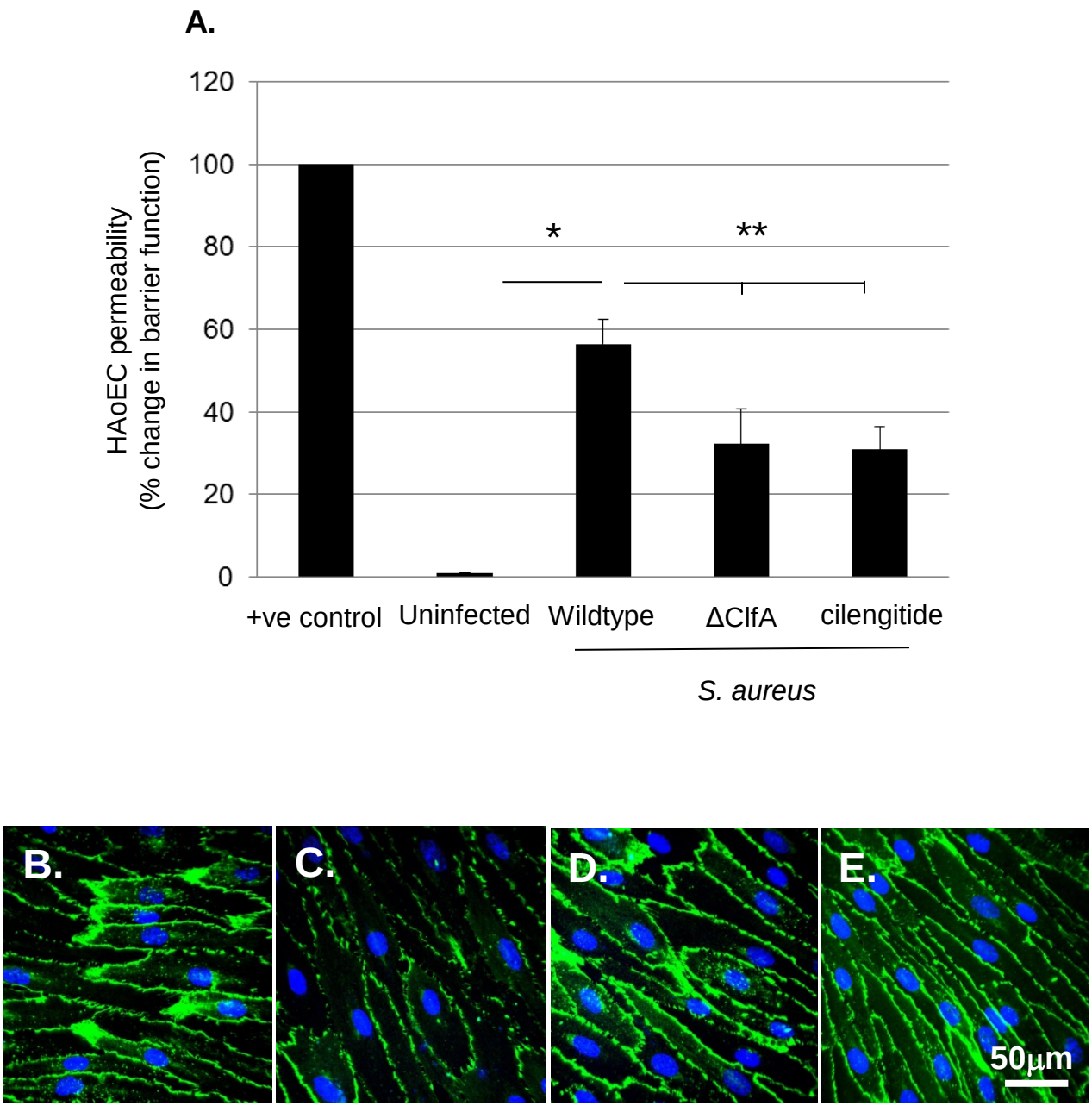
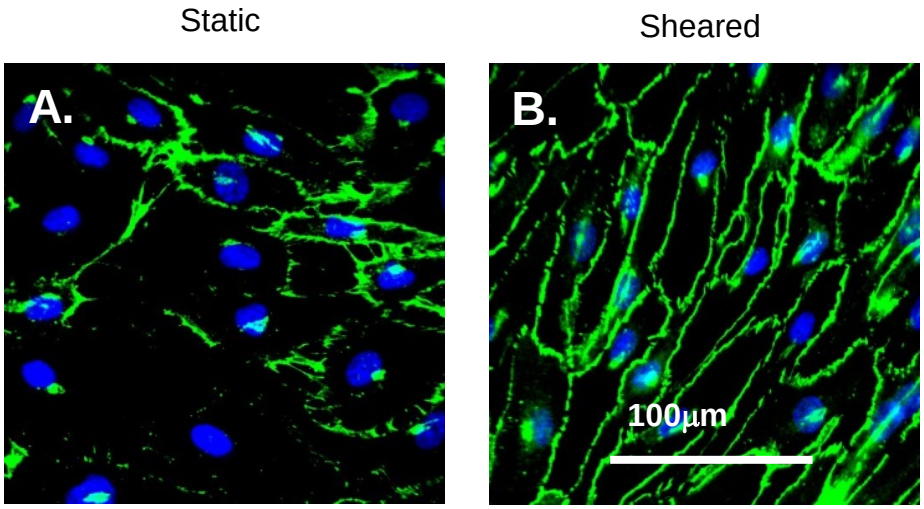

A.
HAoEC proliferation
(number of cells per well x10^5)
20
15
10
5
0
0
24
Time (hrs)
*
**
B.
HAoEC proliferation
(number of cells per well x10^5)
20
15
10
5
0
0
24
Time (hrs)
*
**
C.
Apoptosis
(% positive cells)
60
50
40
30
20
10
0
Uninfected
Wildtype
ΔClfA
S. aureus
*
D.
Apoptosis
(% positive cells)
90
80
70
60
50
40
30
20
10
0
Uninfected
Wildtype
cilengitide
S. aureus
*
**

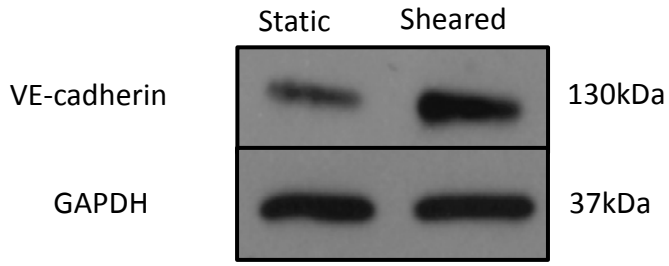
Figure 7.


A.
HAoEC permeability
(% change in barrier function)
120
100
80
60
40
20
0
*
**
+ve control
Uninfected
Wildtype
ΔClfA
cilengitide
S. aureus
B.
C.
D.
E.
50μm

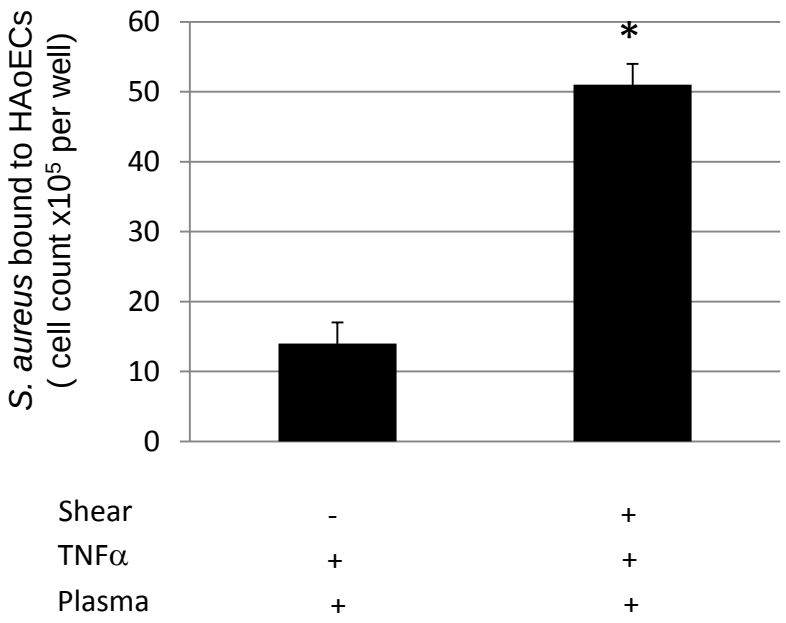
Supplementary figure 1



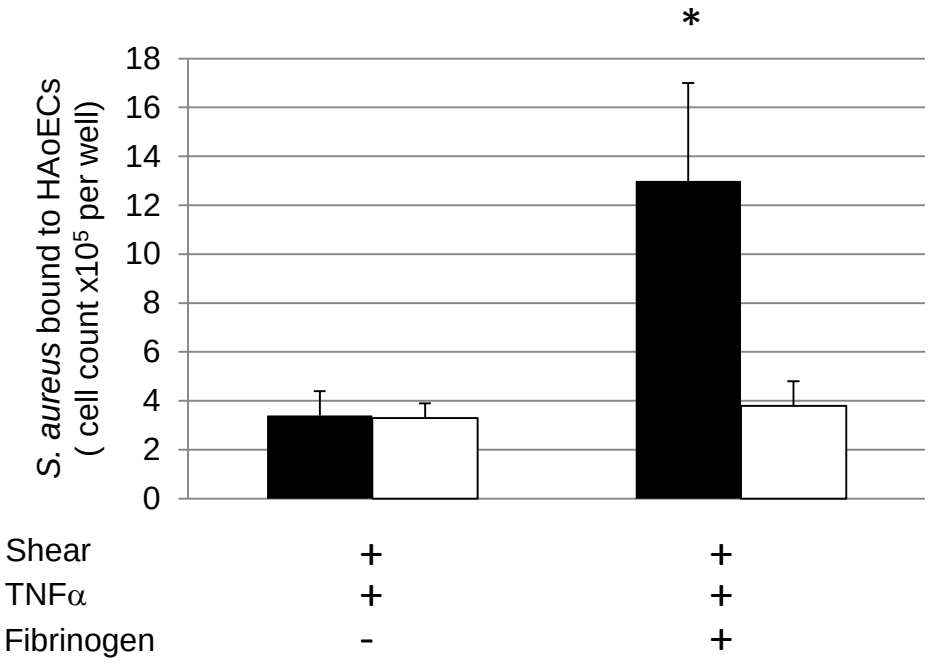
C.



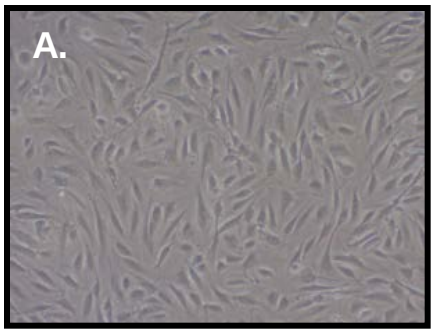
D.

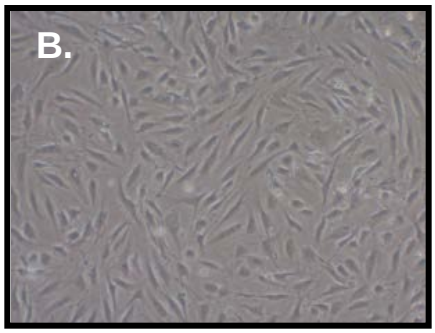


Supplementary figure 2

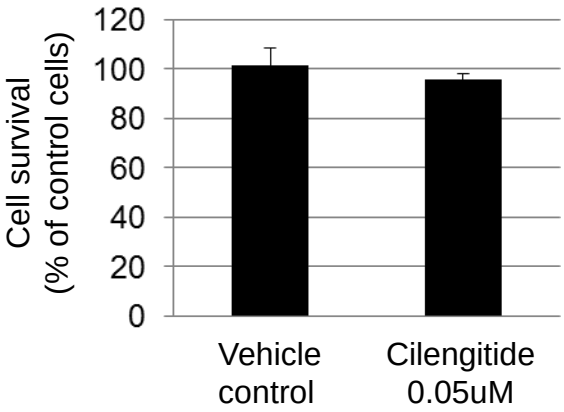


Supplementary figure 3





C.



D.