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ORIGINAL ARTICLE

Inhibition of major integrin $\alpha_V\beta_3$ reduces *Staphylococcus aureus* attachment to sheared human endothelial cells

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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 EC.
- 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.

Summary. *Background:* Vascular endothelial dysfunction with associated edema and organ failure is one of the hallmarks of sepsis. Although a large number of microorganisms can cause sepsis, *Staphylococcus aureus* (*S. aureus*) is one of the primary etiologic agents. Currently, there are no approved specific treatments for sepsis, and the initial management bundle is therefore focused on cardiorespiratory resuscitation and mitigation of the immediate threat of uncontrolled infection. The continuous emergence of antibiotic-resistant strains of bacteria necessitates the development of new therapeutic approaches for this disease. *Objective:* To identify the molecular mechanisms leading to endothelial dysfunction as a result of *S. aureus binding*. *Methods*: Binding of wild type and Clumping factor A (ClfA) deficient *S. aureus* Newman to the endothelium was measured *in vitro* and in the mesenteric circulation of C57Bl/6 mice. The effects of the $\alpha_V\beta_3$ blocker–cilengitide–on bacterial binding, endothelial VE-cadherin expression, apoptosis, proliferation and permeability were assessed. *Results:* The major *S. aureus* cell wall protein ClfA bound to endothelial cell $\alpha_V\beta_3$ in the presence of fibrinogen. This interaction resulted in disturbances in barrier function mediated by VE-cadherin in endothelial cell monolayers, and ultimately cell death by apoptosis. With a low concentration of cilengitide, ClfA binding to $\alpha_V\beta_3$ was significantly inhibited both *in vitro* and *in vivo*. Moreover, preventing *S. aureus* from attaching to $\alpha_V\beta_3$ resulted in a significant reduction in endothelial dysfunction following infection. *Conclusion:* Inhibition of *S. aureus* ClfA binding to endothelial cell $\alpha_V\beta_3$ by cilengitide prevents endothelial dysfunction.

Keywords: $\alpha_V\beta_3$; clumping factor A; endothelial cell; infection; sepsis; *Staphylococcus aureus*; vascular permeability.

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Introduction

Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. There are 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, binding of bacteria to endothelial cells results in activation and granule mobilization. This leads to von Willebrand factor (VWF) deposition on the surfaces of the endothelial cells, which contributes to rapid platelet translocation and thrombus formation [4]. Concomitant decreases in the levels of anticoagulation factors, with a reduction in the thrombomodulin level on the surface of the endothelial cell and a reduction in the circulating level of protein C, leads to clot formation and triggers disseminated intravascular

coagulation [5]. Breakdown of the endothelial barrier results in fluid leakage into the extravascular space, leading to life-threatening edema 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 tissue damage and death [7]. At present, there is no effective specific antisepsis 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 obtained from 14 000 intensive care unit patients in 75 countries suggests that *Staphylococcus aureus (S. 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 attributable, in part, to its expression of a wide array of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) [11]. Using these MSCRAMMs, *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 $\alpha_{IIb}\beta_3$ on platelets and inducing rapid thrombus formation under physiologic conditions *in vitro*. Inhibition of $\alpha_{IIb}\beta_3$ or the use of a strain deficient in expression of ClfA prevents thrombus formation [12]. Integrin $\alpha_{IIb}\beta_3$ is platelet-specific, and is not expressed on endothelial cells; however, another β_3 integrin, $\alpha_V\beta_3$, 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 $\alpha_V\beta_3$ that results in endothelial cell apoptosis and loss of barrier integrity (increased permeability). Furthermore, we identified a compound that inhibits binding of *S. aureus* 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 T. Foster (Trinity College Dublin). Cilengitide was a kind gift from H. Kessler (Technical University of Munchen, Germany).

Blood collection and plasma preparation

Whole blood was collected from healthy donors, and anticoagulated with hirudin (300 U mL^{-1}). Plasma was obtained as described previously [14]. Approval for the collection of whole blood was obtained from the Ethics Committee in the Royal College of Surgeons in Ireland (REC 679b). Informed consent was provided in accordance with the Declaration of Helsinki.

Bacterial growth conditions

The bacterial strains used in this study were: *S. aureus* Newman wild-type (WT) NCTC 8178 [15], *S. aureus* ΔClfA DU5876 (clfA::Erm^R; defective in ClfA) [16], *S. aureus* ΔSpA DU5971 (spa::Ka^R; defective in protein A) [17], *Lactococcus lactis* mock-transfected NZ9800 (pKS80 empty vector) [18], *L. lactis* +ClfA NZ9800 (pNZ8037 ClfA ErmR) [19], *L. lactis* +ClfA PY NZ9800 (pNZ8037::clfa PY Cam^R, expressing ClfA in which Pro336 and Tyr338 are replaced with serine and alanine, respectively) [20], and *L. 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 physiologic state of human blood vessels, primary derived human aortic endothelial cells were cultured and subjected to hemodynamic shear force in endothelial cell growth medium as previously described (Promocell, Heidelberg, Germany) [22]. Unless otherwise stated, endothelial cell infection was preceded by incubation with 10 ng mL^{-1} tumor necrosis factor (TNF)-α for 4 h at 37 °C in 5% CO_2, followed by exposure to plasma, fibrinogen (4 mg mL^{-1}) or IgG (1–8 mg mL^{-1}) for 1 h. In some experiments, cells were preincubated with the $\alpha_V\beta_3$ antagonist cilengitide (0.05 μM) for 1 h between the TNF-α exposure and the addition of plasma/fibrinogen.

Binding assays

Sheared endothelial cells in the presence of TNF-α and plasma/fibrinogen or 40 μg mL^{-1} purified recombinant $\alpha_V\beta_3$ (R&D Systems, Oxon, UK) were immobilized on microtiter plates, blocked with 1% bovine serum albumin for 2 h at 37 °C, and incubated with 1×10^7 SYBR Green II-stained bacteria at a multiplicity of infection of 400 for a further 1 h at 37 °C. In some experiments, cilengitide was preincubated with the cells or purified protein for 1 h prior to addition of the bacteria. Wells were finally washed gently to remove non-adherent bacteria. Fluorescence was read at 485/535 nm in a plate reader (1420 Victor V3; Perkin Elmer, Dublin, Ireland) before (Reading1) and after (Reading2) the final wash. The number of adherent bacteria per well was computed as (Reading2/Reading1) × 1 × 10^7.

$\alpha_v\beta_3$ analysis by flow cytometry

$\alpha_V\beta_3$ expression before and after activation with TNF-α (10 ng mL^{-1} for 4 h) was measured by flow cytometry. Endothelial cells were incubated with anti-$\alpha_V\beta_3$ (LM609) or istoype control. Primary antibodies were incubated with the endothelial cells for 1 h at 37 °C, and then with a fluorescein isothiocyanate (FITC)-labeled secondary antibody in the dark. After 20 min of incubation, the samples were analyzed on a flow cytometer (Becton Dickinson, Oxford, UK) on the Fl-1 channel. Data were analyzed with CELLQUEST software (Becton Dickinson).

Dot blots and western blots

Whole cell dot blotting was performed as previously described [23]. Briefly, a 10-μL 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; 1 : 1000) antibodies. For western blotting, endothelial cells were lysed in RIPA buffer, and proteins were separated on a 10% SDS-PAGE gel. Separated proteins were transferred onto nitrocellulose membranes, blocked for 1 h with 5% milk, and probed with mouse VE-cadherin primary antibody (Santa Cruz, Dallas, TX, USA; 1 : 1000). Anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Biolegend, San Diego, CA, USA,; 1 : 5000) were used as the secondary antibodies. Proteins were detected by the use of enhanced chemiluminescence (Millipore, Watford, UK).

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

Sheared endothelial cells were loaded with the Ca^{2+}-sensitive dye Fluo-4 AM (5 μM; Molecular Probes, Basingstoke, UK) for 20 min. Endothelial cells were then perfused with HEPES buffer containing 10 ng mL^{-1} TNF-α and 0.4 mg mL^{-1} fibrinogen, and switched to the same buffer containing bacteria after 2 min. Samples were excited at 488 nm, and emission at > 500 nm was measured at 2-min intervals. Live images were acquired with an epifluorescence microscope (AxioObserverZ1; Zeiss, Oberkochen, Germany). Time courses 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 mAb (1 : 50; Santa Cruz) or VWF rabbit polyclonal antibody (1 : 50; BD Biosciences, San Jose, CA, USA), and fluoresced by use of a secondary Alexafluor 488 donkey anti-rabbit or donkey anti-mouse antibody (1 : 200; Invitrogen, Waltham, MA. USA). Coverslips were mounted on slides with fluorescent mounting medium containing 4′,6-diamidino-2-phenylindole for staining of nuclei (ProLong; Invitrogen). Control experiments were performed by incubating samples with primary or secondary antibody alone. Images were acquired with an inverted epifluorescence microscope. For extracellular VWF quantification, 10 images were captured per field (METAMORPH, Sunnyvale, CA, USA), background was subtracted, nuclei were counted, and VWF-attributable fluorescence was measured (IMAGEJ; U. S. National Institutes of Health, Bethesda, MD, USA). Extracellular VWF was computed as fluorescence intensity per cell, by 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 hemocytometer in a 1 : 1 dilution with Trypan Blue after a 24-h period, and comparing the count to the seeding density. Apoptosis was determined by flow cytometry. Following the 24-h infection, endothelial cells were trypsinized, washed, and resuspended in ice-cold phosphate-buffered saline. The endothelial cells were pelleted in fluorescence-activated cell sorting tubes, and resuspended in the reagent mix of TACS Annexin V Kits (AMS Biotechnology, Oxford, UK), according to the manufacturer's instructions. Endothelial cells were incubated in the dark at room temperature for 15 min, and analyzed on a flow cytometer (BD FACSCanto II Flow Cytometer; BD Biosciences).

Permeability assay

Endothelial barrier permeability was assessed as previously described [24]. Briefly, sheared endothelial cells were plated at a density of 2×10^5 cells mL^{-1} on the upper chamber of hanging inserts (Millicel, pore size of 0.4 μM; Millipore), and endothelial cell medium was added to the lower chamber. Following infection, FITC–dextran (250 μg mL^{-1}; 40 kDa) was added to the endothelial cells in the upper chamber. Permeability was determined after 24 h by measuring the amount of FITC–dextran that permeated through the endothelial cells into the lower chamber with a fluorescent plate reader (1420 Victor V3; Perkin Elmer). Data are expressed as percentages of 100% permeability.

Mesenteric perfusion model

S. aureus binding to mouse mesenteric endothelium was measured as previously described [4]. Briefly, 6–8-week-old C57Bl/6 mice were fasted and anesthetized with ketamine/xylazine (125/12.5 mg kg^{-1}, intraperitoneal). The endothelium on the mesenteric circulation was exposed, and activated with A23187 (10 mM). Bacteria were labeled with 5 (6)-carboxy-fluorescein *N*-hydroxysuccinimidyl ester, and injected through the jugular catheter. Timelapse images

were acquired with an inverted fluorescence microscope. The fluorescent signal in the blood vessel corresponding to bound bacteria was quantified manually for each frame, and the average was computed as arbitrary fluorescence units. In some experiments, 23.5 ng kg^{-1} cilengitide was administered to the mice for 5 min before the addition of bacteria. Animal experiments were approved by the Ethical Committee of the University of Leuven.

Statistics

Data are presented as mean ± standard error of the mean. Experiments were carried out in triplicate with a minimum of three independent experiments. Statistical differences between groups were assessed by ANOVA with Tukey *post hoc* tests or *t*-tests, as indicated. A *P*-value of < 0.05 was considered to be significant.

Results

S. aureus *binds to endothelial cells grown under hemodynamic 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 10 dyn cm^{-2} for 24 h to mimic the conditions that arterial cells experience *in vivo*. The images showed that endothelial cells grown under static conditions have a random 'cobblestone' morphology and a distinct lack of immunostaining for the adherens junction protein VE-cadherin at the endothelial cell–cell border (Fig. S1A). In contrast, endothelial cells sheared at 10 dyn cm^{-2} aligned in the direction of flow, and there was clear staining for VE-cadherin at the plasma membranes of the endothelial cells at sites of cell–cell contact (Fig. S1B). Consistent with this, statically grown endothelial cells expressed less VE-cadherin than sheared endothelial cells (Fig. S1C). The formation of adherens junctions by VE-cadherin contributes to the functional barrier role of the endothelium [25], and sheared endothelial cells therefore better represent the cellular morphology observed in the vasculature and constitute a more physiologically relevant model with which to study endothelial cell–bacteria interactions.

We observed no significant effect on *S. aureus* binding to sheared endothelial cells when either plasma proteins or TNF-α were added separately. However, addition of human plasma and TNF-α together resulted in a significant increase in *S. aureus* binding to sheared endothelial cells (Fig. 1). Interestingly, we also observed that *S. aureus* binding in the presence of plasma and TNF-α was significantly higher for sheared endothelial cells than statically grown cells (Fig. S1D). On the basis of these observations, unless otherwise stated, all further experiments were performed on sheared endothelial cells in the presence of human plasma/fibrinogen and TNF-α.

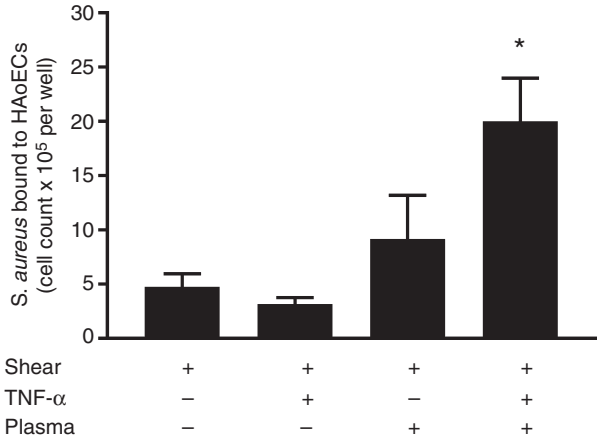

Fig. 1. Sheared endothelial cells constitute a dynamic *ex vivo* model for the study of *Staphylococcus aureus* (*S. aureus*) binding. *S. aureus* adhesion to sheared endothelial cells, tested in the presence and absence of plasma and 10 ng mL^{-1} of tumor necrosis factor (TNF)-α, is shown. Equal amounts of endothelial cells were plated in microtiter plates. After blocking with 1% bovine serum albumin, cells infected with known amounts of SYBR Green I-labeled *S. aureus* Newman (10^7) were added and incubated for 1 h. Total fluorescence was measured on a plate reader at 485/535 nm. Unbound bacteria were removed by gently washing with phosphate-buffered saline, and the fluorescence of attached bacteria was acquired. Results are expressed as number of bacteria attached per well. $*P < 0.05$ versus all other groups. HAoEC, human aortic endothelial cell.

S. aureus *ClfA mediates binding to sheared endothelial cells*

The major *S. aureus* cell wall proteins 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 in binding to endothelial cells. An *S. aureus* mutant defective in expression of SpA (ΔSpA), failed to affect binding to sheared endothelial cells. In contrast, an *S. aureus* strain defective in expression of ClfA (ΔClfA) bound to a significantly lower extent to endothelial cells (Fig. 2A). Lack of expression of either SpA or ClfA on *S. aureus* was confirmed by dot blot analysis (Fig. 2C).

To confirm our findings, ClfA was expressed in the surrogate host *L. lactis*. We chose *L. lactis* because 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 level seen with the mock-transfected control, whereas expression of ClfA resulted in significant binding to endothelial cells (Fig. 2B). Overexpression of SpA and ClfA in the surrogate host *L. lactis* was confirmed by dot blot analysis (Fig. 2D).

Fibrinogen acts as a bridge between ClfA and endothelial cells

S. aureus often binds a plasma protein to 'bridge' the bacterial host cells [27]. Previously, we and others have

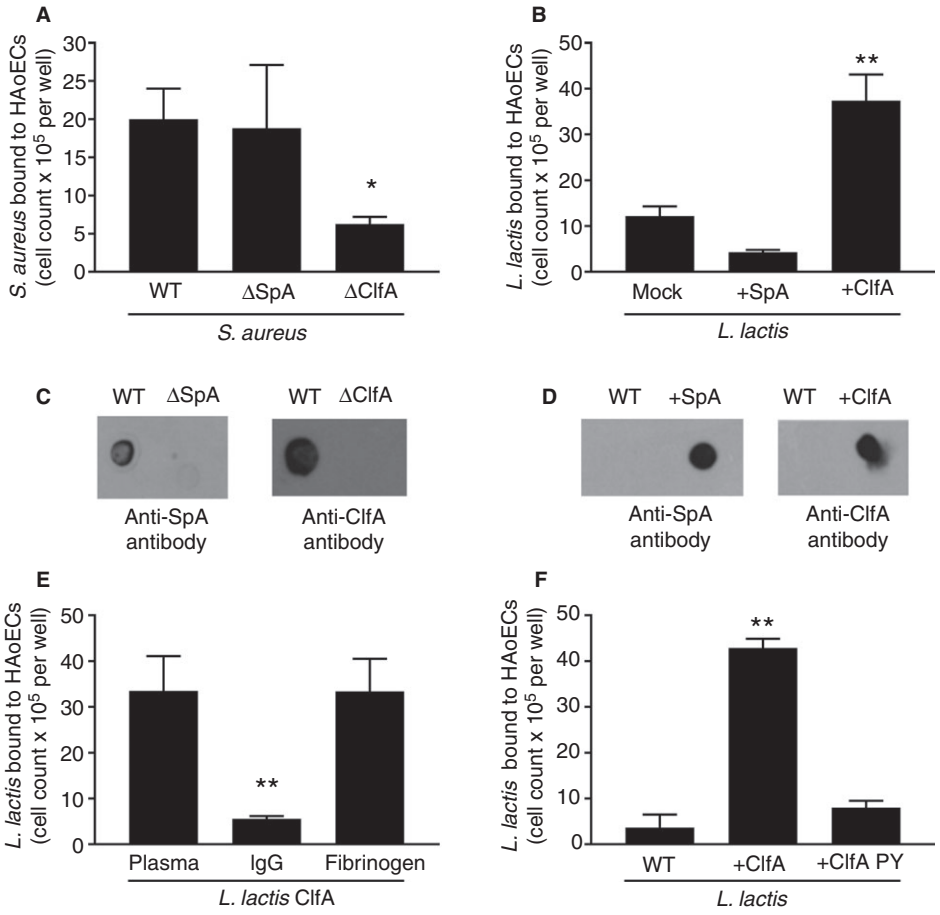

Fig. 2. Clumping factor A (ClfA) binds fibrinogen to bridge *Staphylococcus aureus* (*S. aureus*) to endothelial cells. Binding of bacteria to sheared endothelial cells (10 dyn cm^{-2}; 24 h) was measured at a multiplicity of infection of 400 in the presence of plasma and 10 ng mL^{-1} tumor necrosis factor-α, as previously described. (A) Binding of *S. aureus* wild type (WT) (Newman), deficient in protein A (ΔSpA) or deficient in ClfA (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 analysis. Blots are representative of three independent experiments. (D) Expression of ClfA and SpA on *L. lactis* was confirmed by dot immunoblot analysis of whole bacterial cell lysates. Images are representative of three independent experiments. (E) Effect of the presence of plasma, IgG (1 mg mL^{-1}) or fibrinogen (4 mg mL^{-1}) 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^{-1} fibrinogen. $N = 3$, $^{*}P < 0.05$ versus WT, $^{**}P < 0.05$ versus all other groups, ANOVA. HAoEC, human aortic endothelial cell.

demonstrated that ClfA is capable of binding the plasma proteins IgG and fibrinogen, both individually and simultaneously [12,20]. To determine whether *L. lactis* ClfA binds these plasma proteins, we added purified IgG or fibrinogen to endothelial cells, and then incubated them with *L. lactis* ClfA. Our results showed that IgG (up to 8 mg mL^{-1}) failed to cause significant levels of *L. lactis* ClfA attachment to endothelial cells. In contrast, purified fibrinogen (4 mg mL^{-1}) restored the ability of *L. lactis* ClfA to bind to endothelial cells to levels similar to those observed in the presence of plasma (Fig. 2E). These results are consistent with our findings obtained with *S. aureus*, showing a significant reduction in binding to endothelial cells in the absence of fibrinogen (Fig. S2). Deletion of the amino acids in ClfA (ClfA-PY) that are critical for binding fibrinogen [20] significantly reduced its ability to bind to endothelial cells as compared with *L. lactis* ClfA (Fig. 2F).

S. aureus *ClfA mobilizes* Ca^{2+} *and elicits deposition of VWF onto the surfaces of endothelial cells*

Ca^{2+} is an important second messenger, and its mobilization in endothelial cells results in Weibel–Palade body secretion [28]. VWF is the primary constituent of Weibel–Palade bodies and, once secreted, attaches to the surfaces of endothelial cells, creating a binding site for the *S. aureus* proteins SpA [29] and VWF-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+} levels following *L. lactis* ClfA infection by loading cells with Fluo-4 AM, and surface VWF levels by immunofluorescence. Uninfected endothelial cells showed background levels of Fluo-4 fluorescence over a 12-min period (Fig. 3A,D). Addition of mock-transfected *L. lactis* had no significant effect on baseline Fluo-4

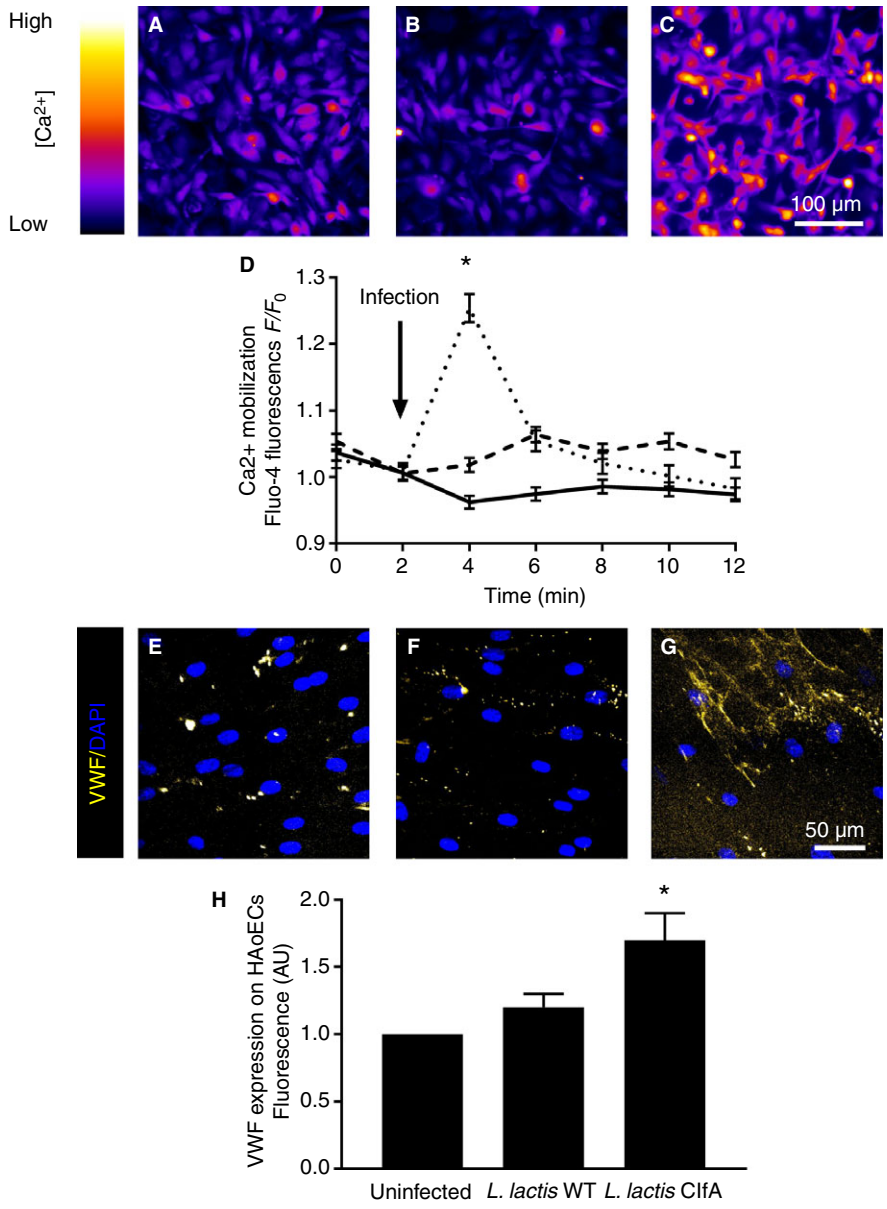

Fig. 3. Clumping factor A (ClfA) induces increases in intracellular Ca^{2+} levels and von Willebrand factor (VWF) deposition the surfaces of endothelial cells. Endothelial cells were sheared for 24 h, trypsinized, and lawned in Ibidi microperfusion chambers. Cells were preloaded with Fluo-4 AM for intracellular Ca^{2+} detection by epifluorescence microscopy under flow (10 dyn cm^{-2}) at 37 °C with a × 40 oil immersion lens objective on a Zeiss AxioObserverZ1 epifluorescence microscope coupled to a CCD camera and equipped with mercury lamp and appropriate filters. The light intensity was adjusted to prevent signal saturation. After an initial 2 min of perfusion with HEPES solution containing 10 ng mL^{-1} tumor necrosis factor (TNF)-α and 4 mg mL^{-1} fibrinogen, the perfusate was switched, in order to induce infection, to the same solution containing $OD_{600\ nm}$ 0.6–0.7 *Lactococcus lactis* wild-type (WT) (n = 8), *L. lactis* ClfA (n = 6), or no bacteria (uninfected control, n = 8). Samples were excited at 488 nm, and emission at > 500 nm was measured at regular time intervals. (A–C) Representative 'fire' pseudo-color images corresponding to (A) uninfected, (B) *L. lactis* WT-infected and (C) *L. lactis* ClfA-infected endothelial cells, acquired 2 min after infection. Scale bar: 50 μm. (D) Fluo-4 semiquantitative time course expressed as F/F_0 (F_0 = at the time of infection) for uninfected (continuous line), *L. lactis* WT-infected (dashed line) and *L. lactis* ClfA-infected (dotted line) endothelial cells. Arrow indicates the start of infection. For VWF measurements, sheared endothelial cells (24 h) were exposed to *L. lactis* WT or *L. lactis* ClfA in the presence of fibrinogen (4 mg mL^{-1}) and TNF-α (10 ng mL^{-1}) for 60 min under 10 dyn cm^{-2} shear. Uninfected cells were used as a control. The same IMAGEJ brightness and contrast settings were applied to all displayed images. Scale bar: 50 μm. (E–G) Representative images of surface VWF (yellow) detected by immunofluorescence of non-permeabilized cells (plan-apochromat × 63/1.40 oil immersion objective (excitation/emission 488 nm/> 505 nm for VWF and 350 nm/> 400 nm for 4′,6-diamidino-2-phenylindole [DAPI]) corresponding to uninfected (E), infected with *L. lactis* WT (F) and infected with *L. lactis* ClfA (G) groups. Nuclei were stained with DAPI (blue). (H) Semiquantitative analysis of VWF levels on the surfaces of endothelial cells, computed as fluorescence per cell (see Materials and methods) (n = 3). *P < 0.05 versus all other groups, ANOVA. AU, arbitrary units; HAoEC, human aortic endothelial cell.

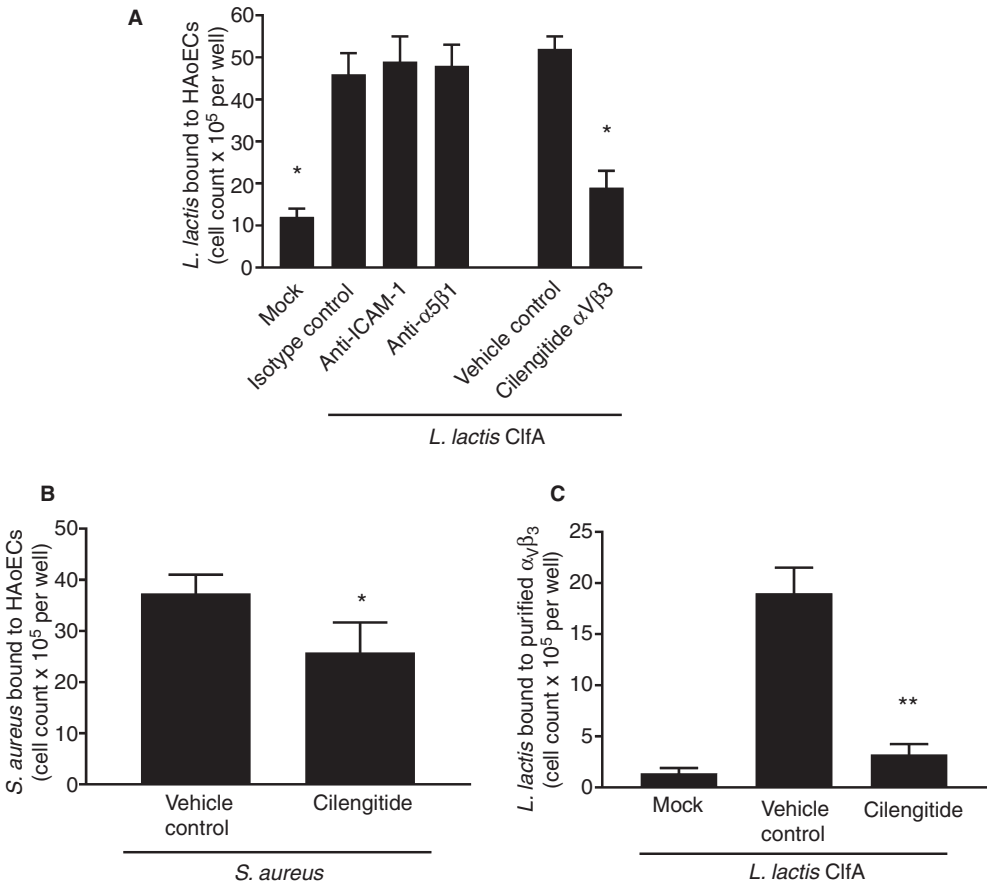

Fig. 4. Fibrinogen bridges clumping factor A (ClfA) to endothelial cell $\alpha_V\beta_3$. (A) Sheared endothelial cells (10 dyn cm^{-2}; 24 h) in the presence of fibrinogen (4 mg mL^{-1}) and tumor necrosis factor-α (10 ng mL^{-1}) were preincubated with either isotype/vehicle control, anti-ICAM-1 (20 μg mL^{-1}), anti-$\alpha_5\beta_1$ (20 μg mL^{-1}) or cilengitide (0.05 μM) for 30 min prior to addition of *Lactococcus lactis* ClfA ($n = 3$, $^*P < 0.05$ versus all other groups, ANOVA). (B) Effect of 0.05 μM cilengitide on *Staphylococcus aureus* (*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 $\alpha_V\beta_3$ integrin blocker cilengitide ($n = 3$, $^{**}P < 0.001$). HAoEC, human aortic endothelial cell.

fluorescence (Fig. 3B,D). Addition of *L. lactis* ClfA resulted in a significant transient increase in Fluo-4 fluorescence in endothelial cells (Fig. 3C,D).

Neither the uninfected nor mock-transfected *L. lactis* resulted in secretion of VWF onto the surfaces of the endothelial cells (Fig. 3E,F). Addition of *L. lactis* ClfA to the endothelial cells elicited significant increases in VWF levels on the surfaces of the cells (Fig. 3G,H).

S. aureus *ClfA binds $\alpha_V\beta_3$ 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, $\alpha_5\beta_1$, and $\alpha_V\beta_3$ [30]. Preincubation of endothelial cells with mAbs against ICAM-1 or $\alpha_5\beta_1$ failed to have any effect on *L. lactis* ClfA binding to endothelial cells (Fig. 4A). Preincubation of endothelial cells with an $\alpha_V\beta_3$ antagonist, cilengitide (0.05 μM), significantly reduced *L. lactis* ClfA binding to endothelial cells as compared with the vehicle control (Fig. 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 were detected on endothelial cells preincubated with cilengitide for 24 h (Fig. S3A–F). Consistent with these findings, cilengitide also significantly inhibited *S. aureus* binding to endothelial cells (Fig. 4B). Preincubation of cilengitide with purified $\alpha_V\beta_3$ in the presence of fibrinogen significantly inhibited *L. lactis* ClfA binding (Fig. 4C). Consistent with the finding that $\alpha_V\beta_3$ is upregulated in sepsis patients [13], we also found that $\alpha_V\beta_3$ expression was increased by 51% on our sheared endothelial cells following TNF-α treatment (Table 1).

To validate our finding that ClfA binds to $\alpha_V\beta_3$ *in vivo*, we used real-time videomicroscopy of the murine splanchnic veins, and demonstrated rapid local accumulation of *S. aureus* on the vessel wall (Fig. 5A,D). Inoculation of *S. aureus* ΔClfA resulted in a significant reduction in the number of bacteria adhering to the vessel wall (Fig. 5B,D).

Table 1 Integrin $\alpha_V\beta_3$ levels are significantly increased on human aortic endothelial cells following infection with *Staphylococcus aureus*

Condition	Anti-$\alpha_V\beta_3$–PE binding (Fl units)	% Increase	*P*-value
Resting (no TNF-α)	22 ± 4	–	–
Activated (with TNF-α)	33 ± 4	51 ± 8	< 0.05

TNF, tumor necrosis factor; PE, phycoerythrin. Expression of $\alpha_V\beta_3$ was measured by flow cytometry in the presence and absence of TNF-α (10 ng mL−1). Sheared endothelial cells were incubated with rabbit polyclonal anti-$\alpha_V\beta_3$ or isotype control. Primary antibodies were incubated with the endothelial cells for 1 h at 37 °C, and then with a fluorescein isothiocyanate-labeled secondary antibody in the dark. $\alpha_V\beta_3$ expression was analyzed on a FACS Calibur flow cytometer (Becton Dickinson, Oxford, UK) on the Fl-1 channel. $P < 0.05$.

Furthermore, cilengitide (0.0005 μM) substantially decreased *S. aureus* attachment to the vessel wall endothelium of the mice (Fig. 5C,E).

S. aureus *ClfA binding to $\alpha_V\beta_3$ inhibits proliferation and induces apoptosis in endothelial cells*

Uninfected sheared endothelial cells proliferated as expected over a period of 24 h; however, addition of *S. aureus* to endothelial cells significantly reduced the rate of proliferation (Fig. 6A). Notably, addition of *S. aureus* ΔClfA (Fig. 6A) or addition of cilengitide (Fig. 6B) resulted in a significantly attenuated effect on endothelial cell proliferation as compared with *S. aureus*.

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

S. aureus *ClfA binding to $\alpha_V\beta_3$ induces increased vascular permeability*

We hypothesized that *S. aureus* ClfA binding to $\alpha_V\beta_3$ generates a signal resulting in an increase in permeability, which is a common feature in patients with sepsis. To test this, we measured the paracellular permeation passage of FITC–dextran 40 kDa 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

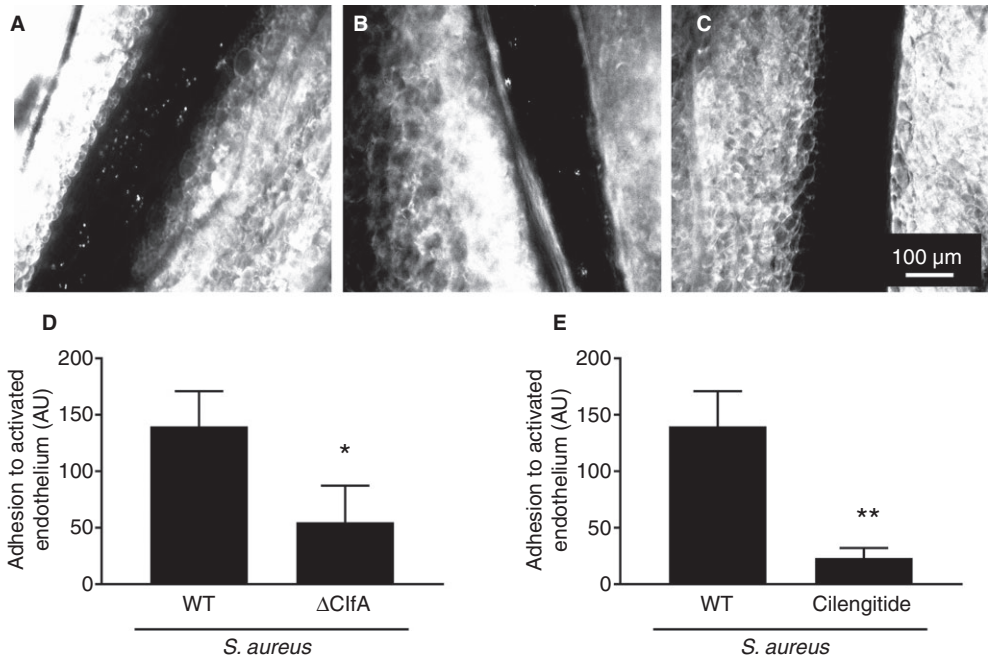

Fig. 5. *Staphylococcus aureus* (*S. aureus*) *in vivo* adhesion to the endothelium is mediated by clumping factor A (ClfA). *S. aureus* bacteria were injected into the right jugular vein of anesthetized mice, and their adhesion to the activated endothelium was measured by timelapse fluorescence recording in the mesenteric veins (one image per second). (A) Adhesion of fluorescent *S. aureus* wild-type (WT) to mesenteric veins. (B, C) Adhesion is diminished when ClfA is knocked out (*S. aureus* ΔClfA) (B), and is almost completely abolished by preadministration of intravenous cilengitide (0.005 μM) (C). Quantitative bacterial adhesion was computed as the average bacterial fluorescence over a period of 40 s (see Materials and methods). (D) Knocking out ClfA in *S. aureus* reduces attachment to the endothelium (n = 10–17, $^*P < 0.05$, t-test). (E) *S. aureus*–endothelium binding is significantly reduced by 0.005 μM cilengitide and ΔClfA (n = 11–17, $^{**}P < 0.005$, t-test). AU, arbitrary units.

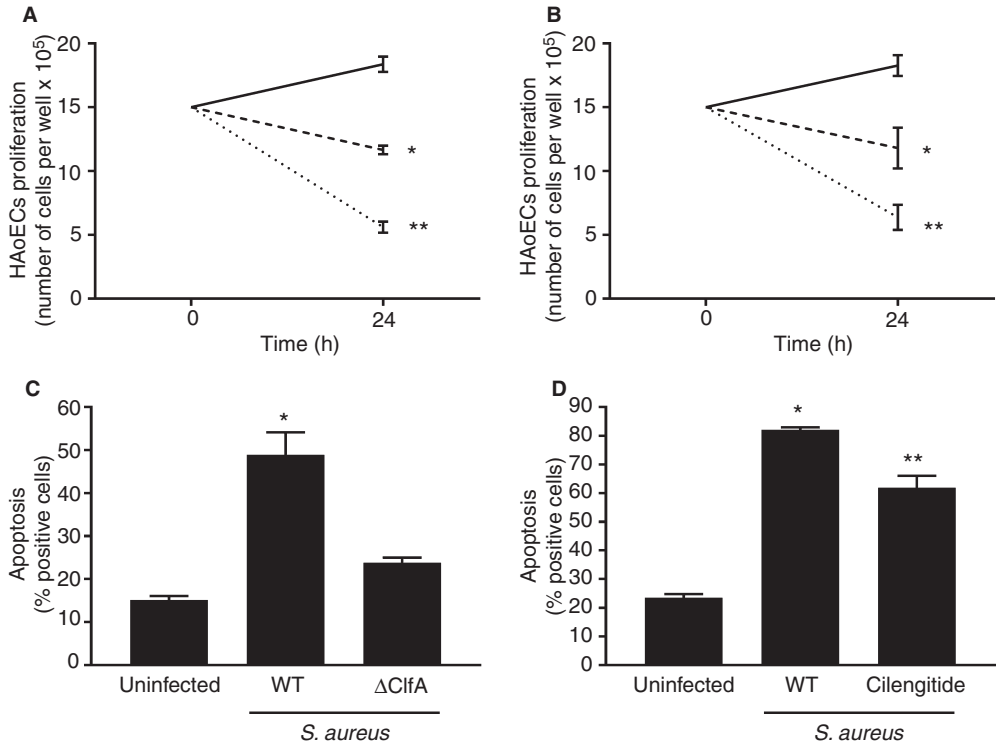

Fig. 6. Loss of endothelial cell proliferation and induction of apoptosis induced by *Staphylococcus aureus* (*S. aureus*) is attenuated by deleting clumping factor A (ClfA) or blocking $\alpha_V\beta_3$ with cilengitide. Cells were seeded at 1.5×10^5 per well (time = 0), and endothelial cell proliferation was evaluated as the cell count after 24 h (time = 24). (A) Endothelial cell proliferation evaluated for uninfected cells (continuous line), and cells infected with *S. aureus* Newman (dotted line) and *S. aureus* ΔClfA (dashed line) ($n = 3$, $^*P < 0.05$ and $^{**}P < 0.05$ versus all other groups, ANOVA). (B) Endothelial cell proliferation evaluated for uninfected cells (continuous line) and cells infected with *S. aureus* Newman (dotted line) and with *S. aureus* Newman in the presence of the $\alpha_V\beta_3$ blocker cilengitide (0.05 μM) (dashed line) ($n = 3$, $^*P < 0.05$ and $^{**}P < 0.05$ versus 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 ($n = 3$, $^*P < 0.05$ versus all other groups). (D) The $\alpha_V\beta_3$ blocker cilengitide (0.05 μM) reduced apoptosis induced by *S. aureus* infection ($n = 3$, $^*P < 0.05$ and $^{**}P < 0.05$ versus all other groups, ANOVA). HAoEC, human aortic endothelial cell; WT, wild-type.

a significant increase in barrier permeability (Fig. 7A). Addition of *S. aureus* ΔClfA failed to increase permeability as compared with WT *S. aureus*. Similarly, inhibition of $\alpha_V\beta_3$ with cilengitide attenuated the increase in permeability induced by WT *S. aureus* (Fig. 7A). Consistent with these results, immunofluorescent staining of VE-cadherin on endothelial cells indicated tight barrier formation in uninfected samples (Fig. 7B). However, on infection with *S. aureus*, VE-cadherin expression in the cell membranes was reduced, suggesting cell–cell detachment (Fig. 7C). In agreement with our previous data, using the ΔClfA strain or treating endothelial cells with cilengitide exhibit attenuated or restored, respectively, VE-cadherin expression and thus barrier integrity compared to the effects of *S. aureus* Newman (Fig. 7D,E).

Discussion

In the present study, we used a dynamic model of endothelial infection that replicates the endothelial conditions experienced during sepsis. Using sheared human endothelial cells, we showed significant *S. aureus* binding in the presence of human plasma and a low level of TNF-α. Attachment was mediated by the *S. aureus* major cell wall protein ClfA, which, in the presence of plasma fibrinogen, bound to $\alpha_V\beta_3$ expressed on human endothelial cells. Binding resulted in Ca^{2+} mobilization, granule exocytosis, and VWF deposition on the surfaces of endothelial cells. Within 24 h of *S. aureus* attachment, there was significant loss of barrier integrity, resulting in increased endothelial permeability. In parallel with the elevated permeability following *S. aureus* infection of endothelial cells, we also observed impaired proliferation in conjunction with elevated apoptosis. Strikingly, the $\alpha_V\beta_3$ antagonist cilengitide significantly reduced all these effects.

S. aureus ClfA is a major MSCRAMM expressed on the surfaces of all naturally occurring *S. aureus* strains, and, most critically, uses fibrinogen to bridge to endothelial cells. Although *S. aureus* expresses other fibrinogen-binding proteins, such as clumping factor B, fibronectin-binding protein A, and fibronectin-binding protein B, we could not detect any contribution of their ability to bind to endothelial cells (data not shown). Although our experimental design allowed us to isolate the *S. aureus*–endothelial cell

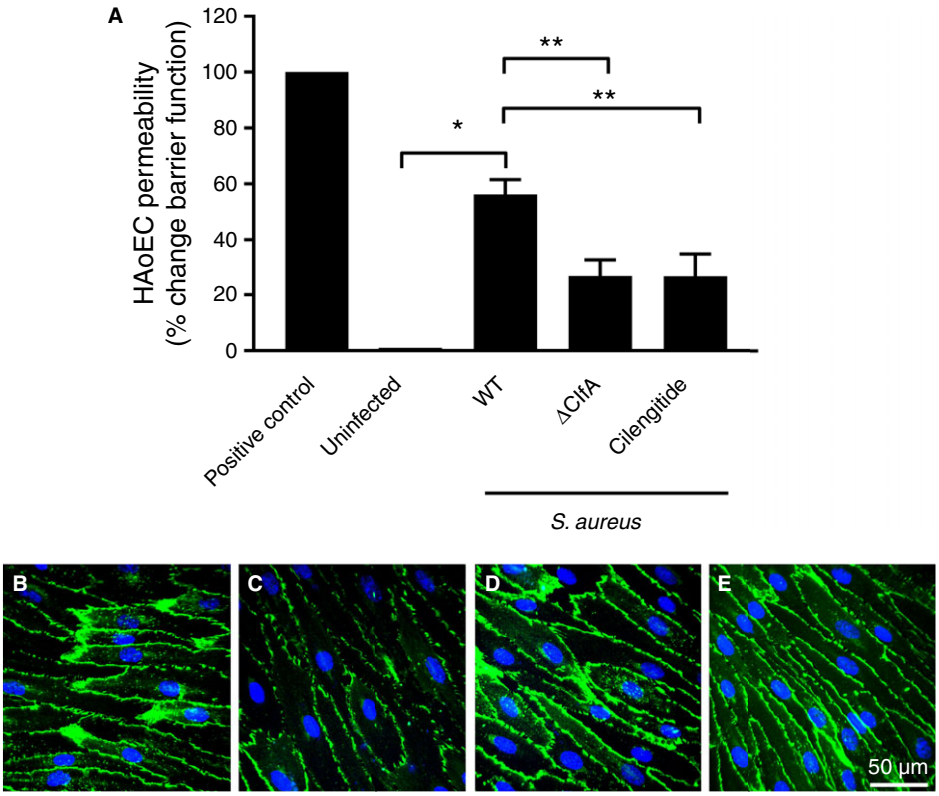

Fig. 7. Increase in endothelial permeability induced by *Staphylococcus aureus* (*S. aureus*) binding through clumping factor A (ClfA) is reduced by blocking $\alpha_V\beta_3$ with a low concentration of cilengitide. (A) The barrier function of endothelial cell monolayers was assessed by measuring the passage of fluorescein isothiocyanate (FITC)–dextran 40 kDa added in the top compartment to the bottom compartment by the use of transwell inserts. Endothelial cells were infected with *S. aureus* Newman or ΔClfA for 24 h (multiplicity of infection of 400). After 24 h, the concentration of FITC–dextran in 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) ($n = 3$, $^*P < 0.05$ and $^{**}P < 0.05$, ANOVA). In order to visualize the expression of VE-cadherin following infection, endothelial cells were fixed, permeabilized, and incubated with antibody against VE-cadherin (primary) and Alexa-fluor 488-conjugated secondary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole. Images were acquired with a fluorescence microscope. (B–E) 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. HAoEC, human aortic endothelial cell; WT, wild-type.

interaction mechanism triggered by ClfA, the participation of other MSCRAMMs in clinical isolates of *S. aureus* cannot be ruled out. Indeed, *S. aureus* ΔClfA showed a residual capacity to bind 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 have demonstrated that *S. aureus* ClfA binding to $\alpha_v\beta_3$ induces Ca^{2+} mobilization and exocytosis of Weibel–Palade bodies, leading to deposition of VWF on the surfaces of endothelial cells. These results suggest that ClfA binding to $\alpha_V\beta_3$ provides the signal that leads to VWF deposition on the surfaces of endothelial cell, and therefore provides a platform for the *S. aureus* VWBP to attach and anchor the bacteria to the vessel wall. Typically, ultralarge VWF multimers deposited on the surfaces of endothelial cells are cleaved by ADAMTS-13 [31]. However, patients with sepsis have an acquired deficiency of ADAMTS-13 that leads to an inability to break down these ultralarge VWF multimers [32], resulting in more *S. aureus* attachment and rapid progression of sepsis. Although 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, facilitating 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 edema in the lungs, kidneys and brains of septic patients [34]. Using a dynamic *ex vivo* model that represents the physiologic 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. 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 have demonstrated that addition of *S. aureus* to the sheared endothelial cells destabilizes the VE-cadherin interactions, leading

to an increase in permeability. Use of a strain deficient in expression of ClfA or blocking $\alpha_V\beta_3$ with cilengitide resulted in a significant reduction in the endothelial cell permeability induced by *S. aureus* and 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 the 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 human umbilical vein endothelial cells resulted in downregulation 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 that used in our study (10 μM versus 0.05 μM, respectively).

Three integrins containing the α_V subunit are expressed in endothelial cells: $\alpha_V\beta_1$, $\alpha_V\beta_3$, and $\alpha_V\beta_5$ [36–38]. Of these, only $\alpha_V\beta_3$ has been reported 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 $\alpha_V\beta_3$ on the endothelium, impeding the activation of injurious pathways resulting in apoptosis and increased vascular permeability. We propose that cilengitide could slow the process of infection progressing to multiorgan 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.

Addendum

P. M. Cummins and S. W. Kerrigan conceived and designed the experiments. C. J. McDonnell, C. D. Garciarena, R. L. Watkin, T. M. McHale, A. McLoughlin, and J. Claes performed the experiments. C. J. McDonnell, C. D. Garciarena, R. L. Watkin, T. M. McHale, A. McLoughlin, J. Claes, P. Verhamme, P. M. Cummins, and S. W. Kerrigan analyzed the data. C. D. Garciarena, P. Verhamme, P. M. Cummins, and S. W. Kerrigan prepared the manuscript.

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Disclosure of Conflict of Interests

S. W. Kerrigan has patent EP16156186 pending. The other authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Development of a dynamic *ex vivo* model that represents the physiologic state of human blood vessels.
Fig. S2. Fibrinogen plays a critical role in the attachment of *S. aureus* to human aortic endothelial cells.
Fig. S3. Cilengitide alone does not induce any toxic effects on human aortic endothelial cells.

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