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BASIC SCIENCE
Valvular heart disease

Staphylococcus aureus endocarditis: distinct mechanisms of bacterial adhesion to damaged and inflamed heart valves

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Aims The pathogenesis of endocarditis is not well understood resulting in unsuccessful attempts at prevention. Clinical observations suggest that *Staphylococcus aureus* infects either damaged or inflamed heart valves. Using a newly developed endocarditis mouse model, we therefore studied the initial adhesion of *S. aureus* in both risk states.

Methods and results Using 3D confocal microscopy, we examined the adhesion of fluorescent *S. aureus* to murine aortic valves. To mimic different risk states we either damaged the valves with a surgically placed catheter or simulated valve inflammation by local endothelium activation. We used von Willebrand factor (VWF) gene-deficient mice, induced platelet and fibrinogen depletion and used several *S. aureus* mutant strains to investigate the contribution of both host and bacterial factors in early bacterial adhesion. Both cardiac valve damage and inflammation predisposed to endocarditis, but by distinct mechanisms. Following valve damage, *S. aureus* adhered directly to VWF and fibrin, deposited on the damaged valve. This was mediated by Sortase A-dependent adhesins such as VWF-binding protein and Clumping factor A. Platelets did not contribute. In contrast, upon cardiac valve inflammation, widespread endothelial activation led to endothelial cell-bound VWF release. This recruited large amounts of platelets, capturing *S. aureus* to the valve surface. Here, neither fibrinogen, nor Sortase A were essential.

Conclusion Cardiac valve damage and inflammation predispose to *S. aureus* endocarditis via distinct mechanisms. These findings may have important implications for the development of new preventive strategies, as some interventions might be effective in one risk state, but not in the other.

Keywords Endocarditis • Platelets • Fibrinogen • von Willebrand factor • *Staphylococcus aureus*

Introduction

With a mortality of 30–40%, *Staphylococcus aureus* endocarditis remains one of the most deadly heart diseases.[1] While important progress has been made on the prevention and treatment of other cardiovascular diseases, the incidence and mortality of *S. aureus* endocarditis has increased over recent years.[2] As we currently fail to grasp the pathogenesis of this enigmatic disease, attempts to prevent endocarditis with antibiotic or anticoagulant prophylaxis or vaccination have all been unsuccessful.[3–5]

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Translational perspective

Staphylococcus aureus endocarditis is a deadly, but understudied heart disease. Owing to our insufficient understanding of its pathogenesis, the occurrence and outcome of endocarditis has not improved over the last decades. Indeed, how bacteria are able to thrive in the turbulent environment of the heart remains elusive. We unravelled some of the mechanisms behind the initial adhesion of *S. aureus* to the heart valves in different clinically relevant risk states using a new unique endocarditis mouse model. These findings are an important step in better understanding endocarditis and a prerequisite for much needed new preventive and therapeutic strategies.

In the early stage of endocarditis bacteria need to adhere to the cardiac valves but are hampered by the high blood flow that rushes over the valve leaflets. This flow creates shear stress, a tangential force that pushes bacteria from the surface.[6] It remains elusive why *S. aureus* is so proficient in overcoming shear stress and in colonizing the heart valves, whilst other frequent causes of bacteraemia seldom cause endocarditis.

As cardiac valves are highly resistant to infection, additional risk factors are needed to provoke endocarditis in case of bacteraemia. It was long thought that mainly heart valve damage predisposes to endocarditis, for example in patients with congenital or rheumatic heart disease.[7,8] Nevertheless, 40% of patients with native valve *S. aureus* endocarditis have structurally normal hearts valves without damage before infection.[9] It was speculated that endocarditis in these patients occurs as a result of cardiac valve inflammation, which is characterized by widespread endothelial cell activation and not by valve damage.[7,8] Unfortunately, current animal models of endocarditis do not reflect the true complexity of this disease and experimental proof of this concept is lacking.

In this article, we studied the initial adhesion of *S. aureus* to damaged and inflamed aortic valves in a new endocarditis mouse model and unravelled the mechanisms that underlie early bacterial adhesion in *S. aureus* endocarditis.

Methods

Bacterial strains

We used *S. aureus* Newman as reference strain. Mutant bacteria deficient in von Willebrand factor (VWF) binding protein (*vwb*), Clumping factor A (*clfA*), Sortase A (*srtA*), staphylocoagulase (*coa*), extracellular adherence protein (*eap*), the accessory gene regulator (*agr*), and *tagO* were created as previously described.[10] USA300 and clinical strains of *S. aureus*, *Staphylococcus epidermidis*, and *Streptococcus gallolyticus* from patient blood cultures were used to validate our findings. Basic phenotypic characterization of the clinical *S. aureus* strain is reported in Supplementary material online, *Table S1*.

Animal experiments

The institutional Ethical Committee approved all animal experiments (license numbers 110/2014, 097/2015, 040/2017). C57Bl/6 wild type (WT) or VWF homozygous knockout ($Vwf^{-/-}$) mice were used, male and female. Anaesthesia procedures are described in the Supplementary material online, *Methods*. The number of animals used is indicated in the figures. Alternatively, each animal is represented by a single dot.

Development of spontaneous endocarditis

To see whether mice spontaneously develop endocarditis, old mice (>18 months of age) and young mice (10–15 weeks of age) were tail vein injected with 2×10^6 CFUs *S. aureus* Newman. Mice were sacrificed 3 days after injection and sections of the aortic valve were stained with Gram staining and analysed for the presence of endocarditis.

Mouse model of early bacterial adhesion

Bacteria were fluorescently labelled with 5(6)-carboxy-fluorescein-N-hydroxysuccinimidylester (Sigma-Aldrich, St. Louis, USA, 30 μg/mL) or Texas Red®-X-succinimidylester (Thermo Fisher, Waltham, USA; 10 μM). Mice were tail vein injected with 3×10^7 CFU fluorescent bacteria, after which a 32-gauge polyurethane catheter (RecathCo, Allison Park, USA) was inserted in the right carotid artery and moved upstream beyond the aortic valve until pulsation of the catheter was detected, assuring its position in the left ventricle.

To generate endothelial damage, the catheter was left in place during 5 (*Figure 2C*) or 15 min (other experiments), damaging the valves with every heartbeat. Afterwards, the catheter was removed. Control mice underwent sham operation.

In contrast, to induce valve inflammation the catheter was placed as described above and was connected to a high-accuracy pump that infused histamine (200 mM, infusion rate 10 μL/min) (Sigma-Aldrich) for 5 min to locally activate the endothelium. In the control group saline was infused. Alternatively, the endothelium was stimulated by *S. aureus* α-toxin (0.5 mg/mL, infusion rate 10 μL/min) (Sigma-Aldrich).

Immediately thereafter, mice were sacrificed and hearts were perfused and fixed. The aortic valve was sectioned in 4–7 cryosections, 200 μm thick, imaged with scanning confocal microscopy (LSM880, Carl-Zeiss) and analysed in 3D with Imaris (Bitplane, Zurich, Switzerland).

For detailed molecular imaging, we injected *in vivo* fluorescent-labelled fibrinogen and platelet staining antibodies and performed immune-fluorescent stainings. Details can be found in the Supplementary material online.

Experimental conditions in early bacterial adhesion

Different isogenic *S. aureus* mutants were compared with WT *S. aureus* in their ability to adhere to inflamed or damaged heart valves. Platelet depletion (>90%) was induced by injection of 12.5 μg of a rat monoclonal antibody against mouse glycoprotein Ibα or an isotype control (Emfret Analytics, Eibelstadt, Germany) 1 h before surgery. Fibrinogen depletion was induced by intravenous injection of six international units of the defibrinogenating agent ancrod (Nordmark, Uetersen, Germany) and confirmed by ELISA (Abcam). To assess the role of surface proteins bacteria were treated with Trypsin-EDTA 0.25% for 30 min.[11]

Long-term endocarditis experiments

Mice were injected with non-fluorescent bacteria (2×10^6–2×10^7 CFU/mouse) and received either cardiac valve damage (catheter present for 30 min) or inflammation (5 min of histamine infusion). Afterwards, the catheter was removed, the carotid artery was ligated and the skin closed. The mice were monitored up to Day 3, when they were sacrificed.

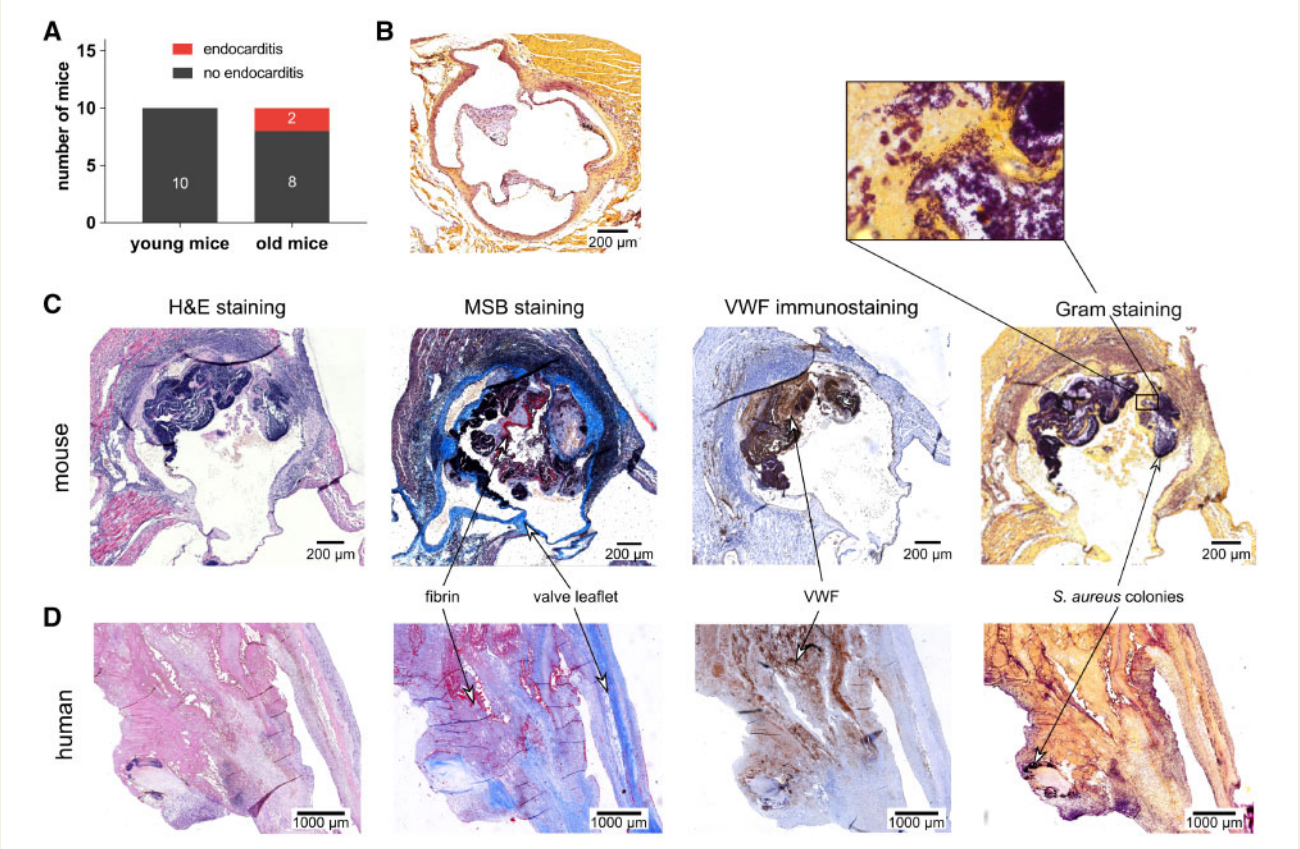


Figure 1 Spontaneous endocarditis in mice (*A*) Proportion of mice that developed endocarditis after injection of 2×10^6 colony forming units *Staphylococcus aureus* Newman in young (10–15 weeks of age) and old mice (>18 months). (*B*) Gram staining of an aortic valve without endocarditis. (*C*) Aortic valve endocarditis in an old mouse. Staining: haematoxylin and eosin (H&E), Martius, Scarlet, and Blue (MSB) (stains fibrin red, collagen blue), von Willebrand factor (VWF) immunostaining, and Gram staining. (*D*) Same stainings of human *S. aureus* endocarditis.

Sections of the aortic valve were stained with Gram staining and analysed for endocarditis.

Statistics

Calculations were done with GraphPad Prism 5.0d (GraphPad Software, La Jolla, USA). The early adhesion measurements were skewed and therefore log transformed, after which the two-tailed Student's *t*-test could be applied. All values are reported as mean ± standard deviation. To test proportions, Fisher's exact test was used. A *P*-value <0.05 was considered significant.

Expanded methods on molecular imaging, electron microscopy, bacterial culture, flow experiments, and animal handling are available in the Supplementary material online.

Results

Mice are suitable as model for endocarditis

We first explored the validity of mice to study infective endocarditis and investigated whether endocarditis spontaneously occurred during bacteraemia. After intravenous injection of 3×10^6 CFUs *S. aureus*, young mice (10–15 weeks old) did not develop endocarditis. However, in old mice (>1.5 years of age) heart valve infection developed in two out of 10 (*Figure 1A*). Comparing human and murine endocarditis, we found remarkable similarities. Both consisted of large bacterial colonies growing unimpeded in a meshwork of platelets and fibrin (*Figure 1B–D*). In both human and murine endocarditis, VWF was abundantly present.

A new mouse model to study the initial adhesion of bacteria to the heart valves in different risk states

To study the initial phase of endocarditis, we generated a mouse model that allowed us to visualize bacterial adhesion to the aortic valve shortly after infection. To this end, we injected mice intravenously with fluorescent-labelled *S. aureus* and quantified bacterial adhesion to the aortic valve using 3D confocal microscopy (*Figure 2A* and *B*, Supplementary material online, *Video S1*). In a first experiment, we mimicked three different clinically relevant risk states (*Figure 2C*). A first group of mice was injected with *S. aureus* but underwent no manipulation of the valves, representative of bacteraemia without additional risk factors. In a second group, the cardiac valves were briefly damaged by the insertion of a catheter trough the aortic valve, which was left in place and used to infuse saline during 5 min. The

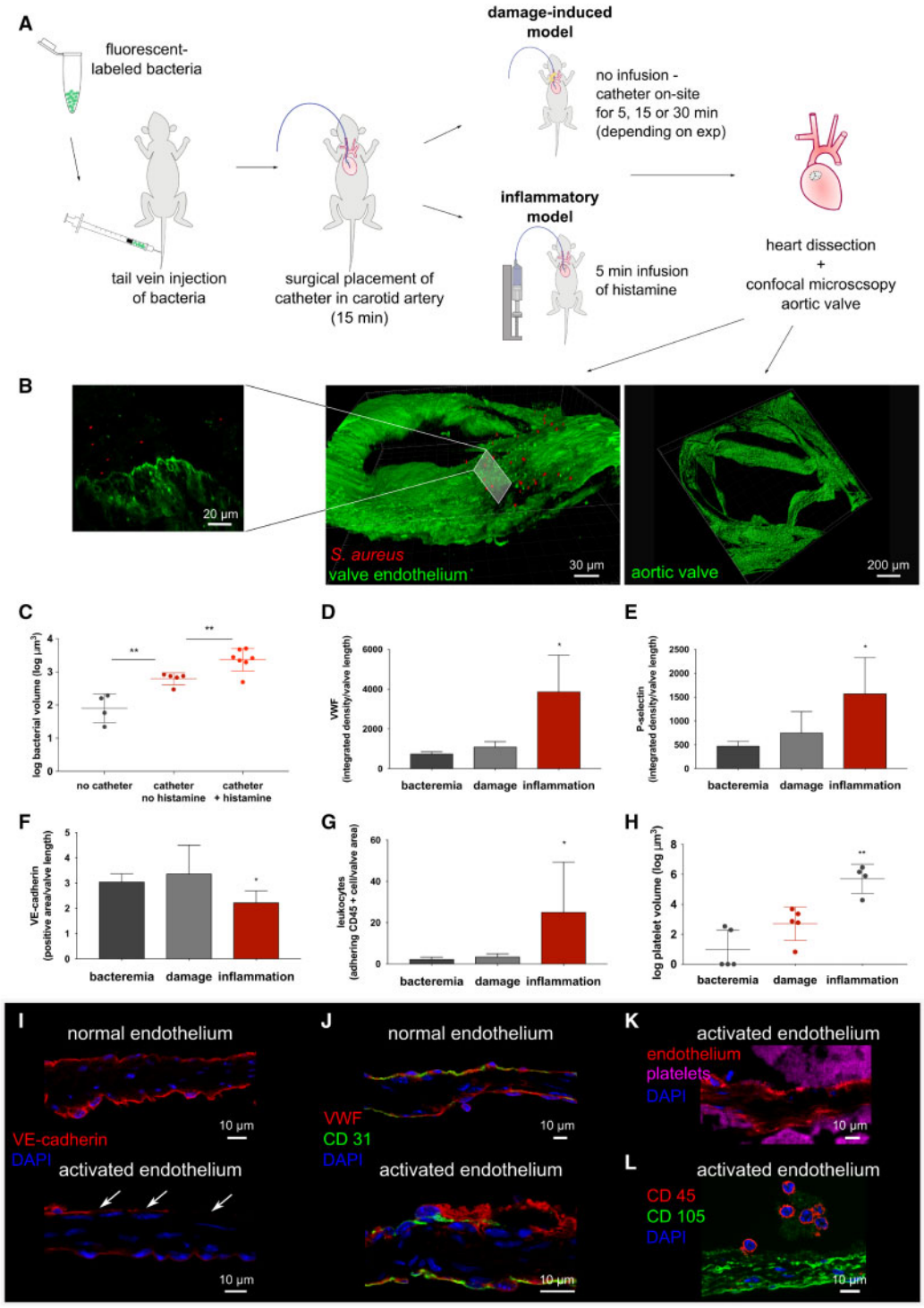


Figure 2 New endocarditis model to measure early bacterial adhesion in different risk states (*A*) Experimental set-up: fluorescent-labelled *Staphylococcus aureus* (3×10^7 CFUs) was injected intravenously in C57Bl/6 mice and a small catheter was inserted in the carotid artery and advanced beyond the aortic valve. To create cardiac valve damage, the catheter was left in place for 5–30 min (depending on the experiment). To mimic cardiac valve inflammation the valvular endothelium was locally activated through infusion of histamine (200 mM at 10 µL/min) for 5 min. Mice were immediately sacrificed and 200 µM thick aortic valve sections were analysed with confocal microscopy. (*B*) 3D reconstruction of an aortic valve (isolectin staining, green), with *S. aureus* adhering (red). (*C*) Quantification of bacterial adhesion in sham operated mice vs. mice with a catheter (5 min damage) vs. mice with catheter + 5 min histamine infusion (inflammation). Results represent log-transformed volumes of single mice. (*D–H*) Immune-fluorescent imaging of aortic valves ($n = 14$) for (*A*) von Willebrand factor (VWF), (*B*) P-selectin, (*C*) VE-cadherin, (*D*) adhering CD45+ cells, (*E*) adhering platelets (stained with anti-Gp1b antibodies). Mean ± standard deviation range is given $^*P < 0.05$, $^{**}P < 0.01$, two-tailed Student;s *t*-test. (*G–J*) Representative images of (*G*) VE-cadherin, (*H*) VWF/CD31, (*I*) platelets, and (*J*) CD45/CD105 stainings.

third group also received a catheter (thus also some cardiac valve damage), but the catheter was used to infuse histamine, a mediator of inflammation and a potent endothelial cell activator.

In the bacteraemia group, hardly any bacteria adhered (*Figure 2C*). However, in mice where the cardiac valves were briefly damaged, a significant increase in bacterial adhesion was seen ($P < 0.01$; *Figure 2C*). Furthermore, the longer the catheter remained in place, the stronger the increase in bacterial adhesion (Supplementary material online, *Figure S1*).

In the third group in which histamine was infused, bacterial adhesion increased even further ($P < 0.01$; *Figure 2C*), suggesting that both cardiac valve damage and inflammation promote bacterial adhesion independently and additively. These results were confirmed with the methicillin resistant (MRSA) USA300 strain and a clinical endocarditis isolate (Supplementary material online, *Figure S2*).

Additional immune-fluorescent stainings confirmed the presence of rapid endothelial cell activation in the inflammation group by showing a significant increase of externally retained endothelial VWF, an increased P-selectin secretion and a decrease in membrane VE-cadherin (*Figure 2D–F, I* and *J*). These changes were absent in the damage group. In addition, infusing histamine led to the recruitment of leucocytes and platelets to the activated endothelium (*Figure 2G* and *H, K* and *L*), which confirms it as an inflammatory condition. Histamine infusion only caused local endothelial activation, as plasma P-selectin levels remained similar 15 and 75 min after surgery (Supplementary material online, *Figure S3*).

From early bacterial adhesion to mature endocarditis

Next, we tested whether this observed early bacterial adhesion was indeed the first step in the development of mature endocarditis. To this end, we intravenously injected *S. aureus* Newman, USA 300, or a clinical endocarditis strain and either damaged the valves with a catheter for 30 min or induced inflammation by 5 min of histamine infusion. After removal of the catheter, the mice were monitored for the development of endocarditis. Control mice underwent sham operation (damage-induced endocarditis) or a saline instead of histamine infusion (inflammation-induced endocarditis).

In the inflammation model using 2×10^6 CFUs *S. aureus* we found no endocarditis in the control group, but a significant proportion of mice with histamine infusion developed endocarditis; 6/17 (24%, $P < 0.05$) for *S. aureus* Newman, 4/12 (33%, $P = 0.09$) for USA300, and 7/18 (39%, $P < 0.01$) for a clinical endocarditis strain (*Figure 3A–C*).

In the damage-induced model, with 2×10^6 CFUs, few mice developed endocarditis after valve damage, but none in the sham group; 0/5 (0%, $P > 0.05$) for *S. aureus* Newman, 1/12 (8%, $P > 0.05$) for USA300, and 1/11 (9%, $P > 0.05$) for the clinical strain (*Figure 3D–F*). However, with a bacterial load of 2×10^7 CFUs, a larger proportion of mice in the damaged, but not in the control group developed endocarditis: 2/6 (33%, $P = 0.45$) for *S. aureus* Newman, 1/12 (8%, $P = 0.9$) for USA300, and 8/10 (80%, $P < 0.01$) for the clinical strain (*Figure 3G–I*).

Using light microscopy we captured the different stages in the development of these endocarditis lesions (*Figure 3J*), observing how early lesions would grow to large vegetations that ultimately destroyed the aortic valve. Importantly, these experimental vegetations looked very similar to those spontaneously occurring in older mice.

In addition, detailed scanning electron microscopy revealed individual staphylococci in large destructive vegetations consisting of platelets and fibrin (*Figure 3K*). Echocardiography confirmed that these vegetations originated from the aortic valve and could cause severe aortic regurgitation (*Figure 3L*).

Mechanisms of bacterial adhesion in damage-induced endocarditis

We then determined the bacterial virulence factors and host factors that mediate early bacterial adhesion, first in the cardiac valve model.

As we previously showed that *S. aureus* overcomes shear stress by binding to VWF, using its VWF-binding protein (vWbp),[10] we hypothesized a role for VWF in early endocarditis. Indeed, when we compared WT mice with VWF knockout mice ($Vwf^{-/-}$), bacteria adhered less well to damaged aortic valves of $Vwf^{-/-}$ mice ($P < 0.05$; *Figure 4A*). To assess whether this reduction was caused by reduced platelet adhesion, we performed antibody-mediated platelet depletion prior to inoculation. However, no difference in bacterial adhesion was found for control and platelet depleted mice ($P = 0.54$; *Figure 4B*). Fibrin(ogen) depletion on the other hand did hamper bacterial adhesion, as mice treated with ancrod, a defibrinogenating agent, showed significantly reduced bacterial adhesion ($P < 0.05$; *Figure 4C*).

Subsequently, we used different isogenic *S. aureus* mutants to investigate which bacterial virulence factors are involved in bacterial adhesion to damaged valves. We hypothesized a central role for the cell wall anchored *S. aureus* adhesins and therefore evaluated sortase A (*srtA*) mutants, as SrtA is responsible for covalently anchoring 24 adhesins to the cell wall. Indeed, compared with WT bacteria, the *srtA* mutant was significantly hampered in its adhesion to the damaged aortic valves compared with WT bacteria ($P < 0.05$; *Figure 4D*). When testing individual adhesins, we found that bacteria lacking *vwb* or clumping factor A (*clfA*), one of the fibrin(ogen) binding proteins also showed decreased adhesion ($P < 0.05$; *Figures 4E* and *F*).

Mechanisms of bacterial adhesion in inflammation-induced endocarditis

Next, we investigated if the same factors contributed to bacterial adhesion after histamine infusion. Again, we found a significant reduction in bacterial adhesion in $Vwf^{-/-}$ mice compared with WT mice ($P < 0.05$; *Figure 5A*). In contrast to the observation in the damage model, platelet depletion did significantly reduce adhesion ($P < 0.001$; *Figure 5B*). Fibrin(ogen) depletion with ancrod on the other hand had no effect on bacterial binding ($P = 0.10$; *Figure 5C*).

Also in the contribution of *S. aureus* virulence factors, substantial differences between damage and inflammation-induced endocarditis were uncovered. Surprisingly, in inflammation-induced endocarditis we found no difference in adhesion between *srtA* mutants and WT *S. aureus* ($P = 0.77$; *Figure 5D*). Also, *vwb* ($P = 0.90$; *Figure 5E*) or *clfA* ($P = 0.55$; *Figure 5F*) mutants adhered similarly.

To corroborate that platelets enable bacterial adhesion in an SrtA independent manner, we performed flow chamber perfusion experiments *in vitro*. WT *S. aureus* or *srtA* mutants were perfused over

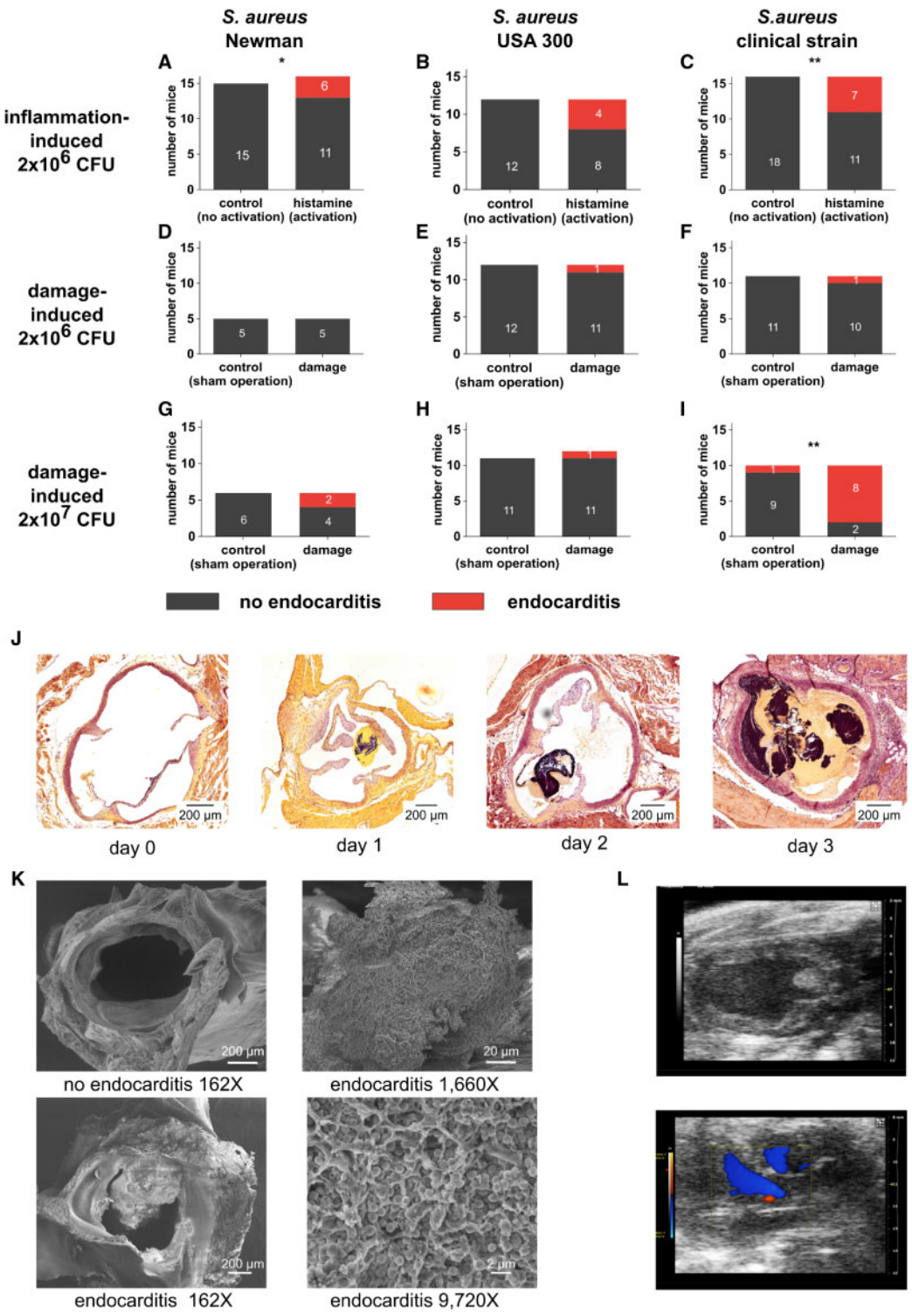


Figure 3 Studying mature endocarditis vegetations. *Staphylococcus aureus* was injected intravenously in C57Bl/6 mice and cardiac valve damage (30 min) or inflammation (5 min histamine infusion) was induced. Afterwards, the catheter was removed and mice were monitored for 3 days to see if endocarditis developed. (*A–I*) Proportion of mice developing endocarditis after infection with three different strains: *S. aureus* Newman, USA 300, and a clinical endocarditis strain; (*A–C*) in the inflammation-induced model with 2×10^6 CFUs, (*D–F*) in the damaged-induced model with 2×10^6 CFUs and (*G–I*) in the damaged-induced model with 2×10^7 CFUs. $^*P < 0.05$, $^{**}P < 0.01$, Fisher's exact. Number of mice used is indicated. (*J*) Gram staining of the aortic valve showing growing vegetations at Day 0–3 after surgery (*S. aureus* Newman). (*K*) Scanning electron microscopy of aortic valve endocarditis with *S. aureus* Newman. (*L*) Echocardiographic imaging of endocarditis lesion causing severe aortic regurgitation (*S. aureus* Newman).

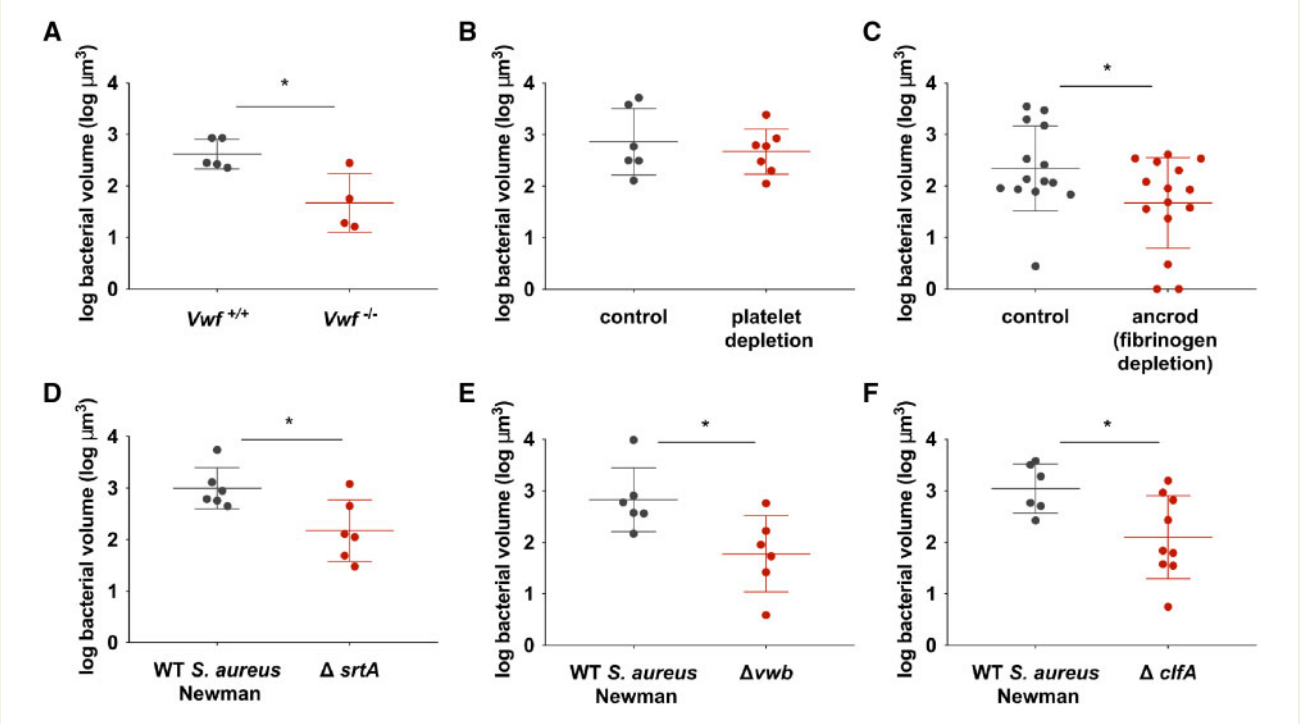


Figure 4 Mechanisms of bacterial adhesion in the damage-induced endocarditis model (15 min of damage by a transaortic catheter). (*A*) Adhesion of *Staphylococcus aureus* Newman in wild type ($Vwf^{+/+}$) vs. VWF knockout mice ($Vwf^{-/-}$), (*B*) in platelet depleted vs. control mice, and (*C*) in ancrod treated vs. control mice. (*D–F*) Adhesion of different *S. aureus* Newman mutants to damage aortic valves compared with wild type bacteria; (*D*) Sortase A (Δ*srtA*), (*E*) von Willebrand Factor binding protein (Δ*vwb*), and (*F*) Clumping factor A (Δ*clfA*). Results represent log-transformed volumes in single mice. Mean ± standard deviation, $*P < 0.05$, two-tailed Student's *t*-test.

resting or activated human endothelial cells activated human umbilical vein endothelial cells (HUVECs) in the presence or absence of platelets (*Figure 5G*). As previously demonstrated, binding of WT *S. aureus* to HUVECs increases strongly if the endothelial cells were activated.[10] However, *SrtA* mutants were unable to bind to activated cells in the absence of platelets, but regained binding capacity when platelets were present (*Figure 5G*). This confirms that platelets can bridge *S. aureus* to the endothelium in an SrtA-independent manner. The Supplementary material online, *Result* section and *Figure S4* describes additional experiments elucidating the bacterial virulence factors involved in this process. However, we were unable to deceiver the exact mechanism.

Finally, because *S. aureus* α-toxin is also known to induce endothelial cell activation,[12] we tested whether *S. aureus* can increase its adhesion by secreting α-toxin. Indeed, when α-toxin was infused, bacterial adhesion to the aortic valves increased significantly compared with saline infusion (*Figure 8F*).

Damage- vs. inflammation-induced endocarditis: detailed imaging

The results above indicate that distinct mechanisms govern bacterial adhesion in damage and inflammation-induced endocarditis. To corroborate these findings, we injected fluorescent-labelled fibrinogen, bacteria and platelet staining antibodies *in vivo*, counterstained the endothelium and made detailed 3D reconstructions of these early endocarditis lesions with confocal microscopy (*Figure 6*, Supplementary material online, *Videos S2* and *S3* for 3D-animations).

In damage-induced endocarditis, the endothelium appeared disrupted and covered with a layer of fibrin (*Figure 6A* and *B*, Supplementary material online, *Video S2*). Bacteria preferentially adhered to this layer, close to the endothelial surface. In addition, platelets were deposited onto the fibrin layer, but only a small fraction of the bacteria were embedded in platelet aggregates.

In the inflammatory pathway on the other hand, endothelial cell activation prompted the adhesion of large amounts of platelets to the valve surface (*Figure 6C* and *D*, Supplementary material online, *Video S3*). *Staphylococcus aureus* was entrapped within the platelet aggregates. Little fibrin deposition was seen directly onto the valve, but in some areas within the freshly adhered platelet clot, coagulation was also initiated.

Lastly, we performed scanning electron microscopy of early endocarditis lesions (*Figure 7*). In case of valve damage we observed fibrin deposition on the damaged valve surface, with bacteria and secondary platelets adhering to the fibrin layer, although it was impossible to discriminate between bacteria and non-activated platelets (*Figure 7A–C*).

After cardiac valve inflammation, there were also small foci of valve damage. However, most abundantly, large platelet thrombi adhered to intact endothelium (*Figure 7D–I*), with freshly activated platelets and neutrophils being incorporated into the growing vegetation.

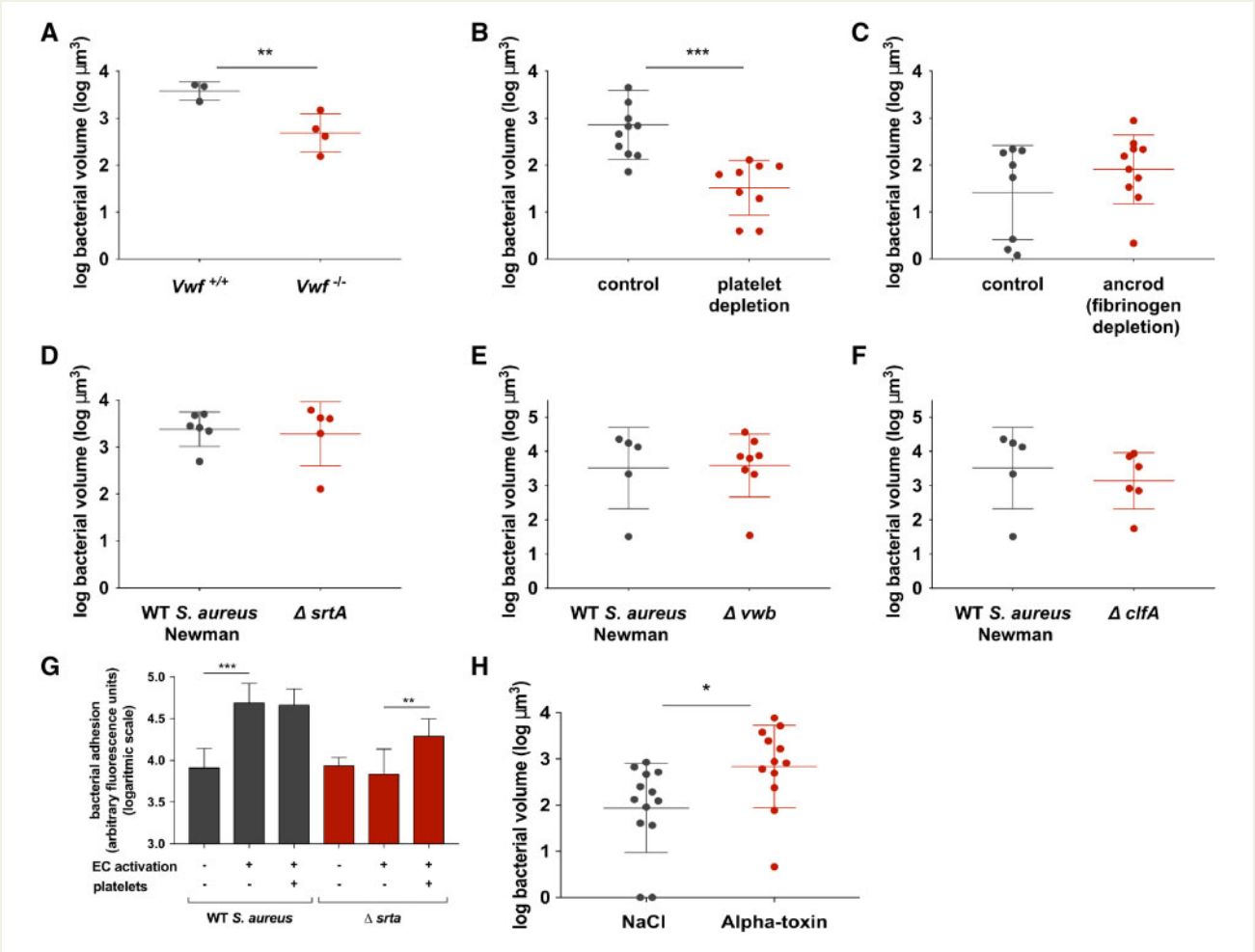


Figure 5 Mechanisms of bacterial adhesion in the inflammation-induced endocarditis model (5 min of histamine infusion through transaortic catheter). (*A*) Adhesion of *Staphylococcus aureus* Newman in wild type ($Vwf^{+/+}$) vs. VWF knockout mice ($Vwf^{-/-}$), (*B*) in platelet depleted, and (*C*) in ancrod treated mice. (*D–F*) Adhesion of different *S. aureus* Newman mutants to damage aortic valves compared with wild type (WT) *S. aureus*; (*D*) Sortase A (Δ*srtA*), (*E*) von Willebrand Factor binding protein (Δ*vwb*), and (*F*) Clumping factor A (Δ*clfA*). Results represent log-transformed volumes in single mice. (*G*) Flow chamber experiment measuring the adhesion of WT *S. aureus* Newman, or Δ*srtA* to resting or activated endothelial cells (shear rate 1000 s^{-1}) in the presence or absence of platelets. (*H*) Adhesion of WT *S. aureus* Newman to aortic valves of mice after local infusion via an aortic catheter of saline or *S. aureus* alpha-toxin (0.5 mg/mL). Mean ± standard deviation, **P* < 0.05, ****P* < 0.005, two-tailed unpaired Student's *t*-test.

Discussion

We studied for the first time the initial stages of *S. aureus* endocarditis using a newly developed unique mouse model. This model allowed us to mimic different clinically relevant risk states for endocarditis and confirmed that not only cardiac valve damage but also valve inflammation and endothelial cell activation predispose for endocarditis. Surprisingly, the mechanisms by which cardiac valve damage and inflammation facilitate bacterial adhesion are different.

A crucial first step for bacteria in infecting the heart valves is withstanding the shear forces created by the high blood flow in the heart. In case of arterial bleeding or thrombosis platelets overcome shear stress by binding to VWF. Remarkably, we found that the same mechanism is at play in the early phase of endocarditis. Indeed, VWF was abundantly present in endocarditis lesions and VWF proved to be crucial in *S. aureus* adhesion to both damaged and inflamed heart valves. Surprisingly, however, VWF mediates bacterial adhesion in different ways depending on the risk state. In damaged-induced endocarditis, circulating VWF is deposited onto the subendothelial matrix and *S. aureus* overcomes shear stress by binding directly to VWF using its VWF binding adhesin vWbp. In inflammation-induced endocarditis, VWF is released by widespread endothelial cell activation, resulting in the adhesion of large amounts of platelets to the valve surface. In this case, *S. aureus* adheres via platelets and uses platelets as a bridge to overcome shear stress. Direct binding to VWF plays only a minor role in this process.

Two mechanisms of early bacterial adhesion in endocarditis therefore emerge (*Figure 7L*). In damaged-induced endocarditis, valvular injury exposes the subendothelium, leading to local fibrin and VWF deposition, to which *S. aureus* can directly adhere using adhesins such as vWbp and Clfa. Platelets are also deposited onto the surface of the heart valve, but contribute less to bacterial adhesion. Cardiac

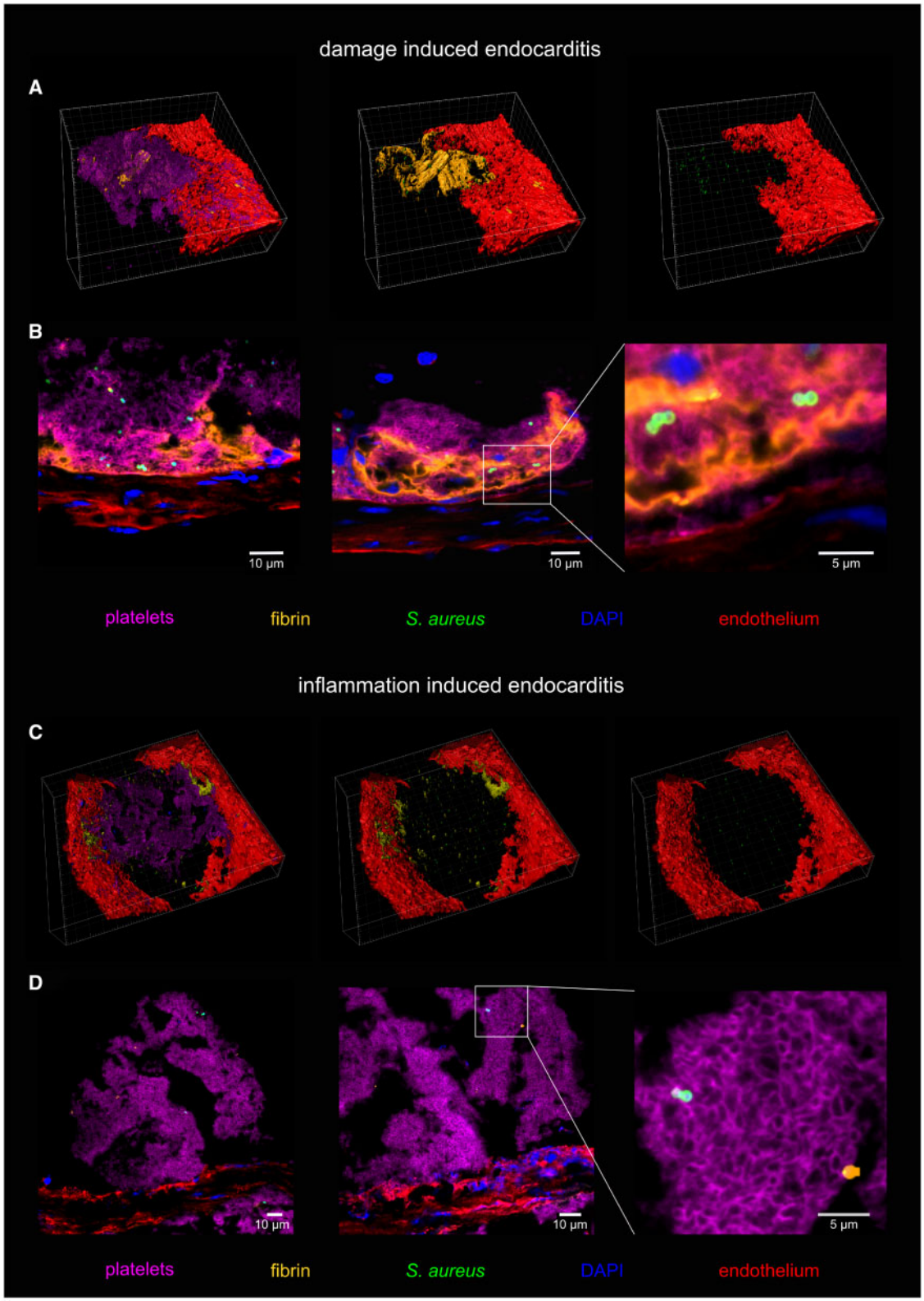


Figure 6 Detailed confocal imaging of early endocarditis lesions. Fluorescent-labelled *Staphylococcus aureus* Newman, fibrinogen, and platelet-staining antibodies were injected *in vivo* and the endothelium was counterstained with isolectin and DAPI. (*A* and *B*) Damage induced endocarditis, with (*A*) 3D reconstruction of an early endocarditis lesion. Different pictures show the same lesion: first panel showing all components, second after removing the platelet layer, and the third after removal of fibrin layer showing only bacteria and endothelium. (*B*) Detailed 2D microscopy images. (*C* and *D*) Inflammation-induced endocarditis: (*C*) 3D reconstruction, (*D*) 2D images.

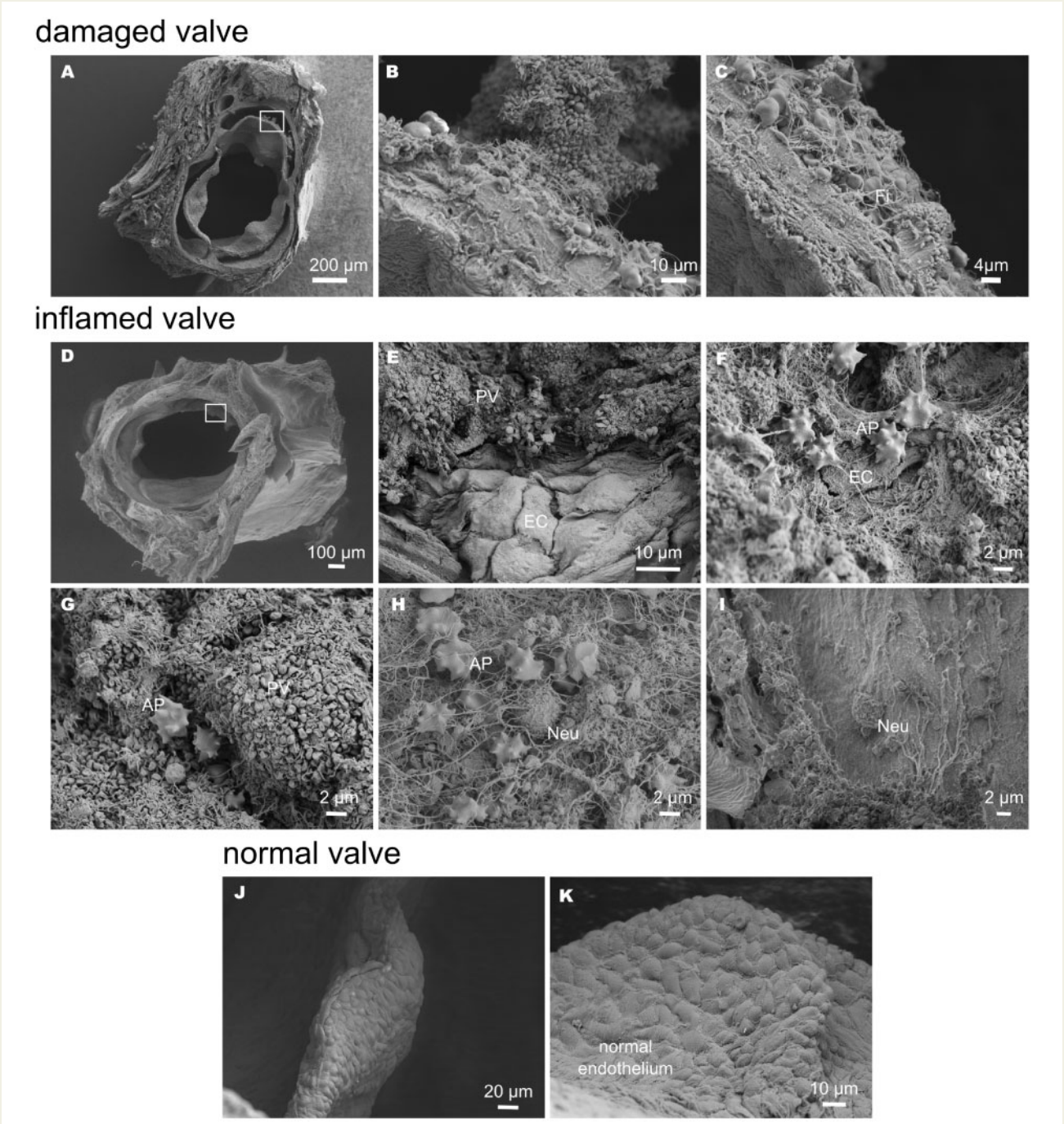
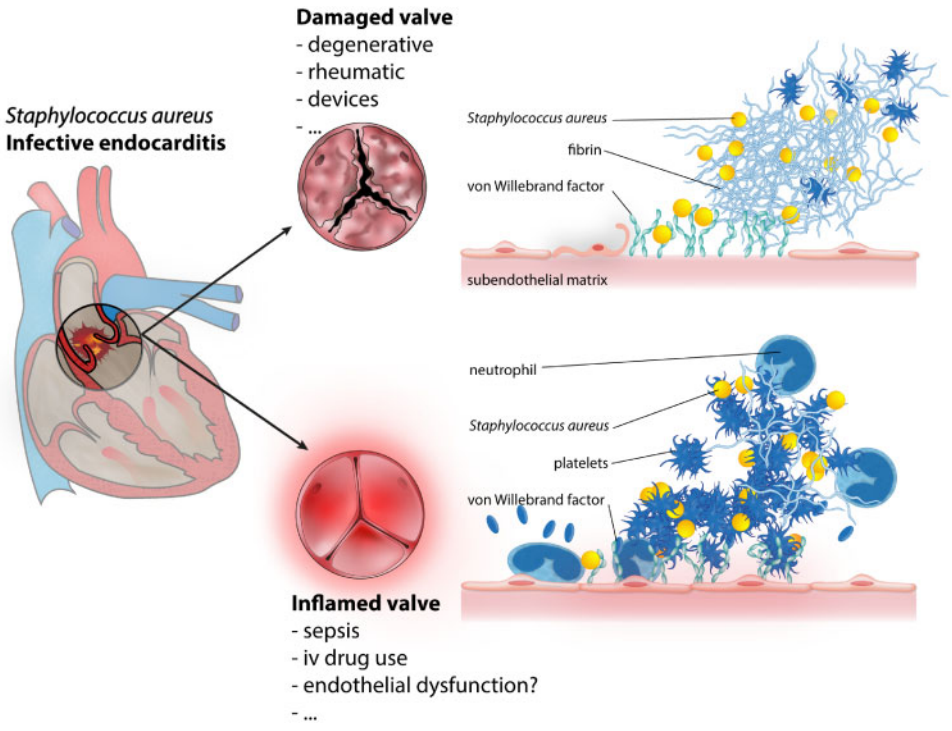


Figure 7 Scanning electron microscopy of early endocarditis lesions. (*A–C*) Early lesion on a damaged aortic valve, showing fibrin (Fi) deposition on damaged endothelium and secondary platelet and bacterial adhesion. (*D–I*) Early lesion on an inflamed heart valve, showing (*E*) a platelet rich vegetation (PV) that adheres to intact endothelial cells (EC). (*F–G*) Activated platelets (AP) are incorporated into the vegetation. (*H* and *I*) Neutrophils (Neu) adhere to the activated endothelium. (*J* and *K*) Normal valves.

valve damage is prominent in case of rheumatic and congenital valve disease, where turbulent blood flow damages the endothelium.

In cardiac valve inflammation, on the other hand, bacterial adhesion is mainly mediated by platelets. Cardiac valve inflammation probably predominates in patients with structurally normal heart valves but who develop endocarditis because they are in an inflammatory state. Examples include intensive care patients who develop an *S. aureus* catheter infection, or IV drug users, which are mostly young people without valve abnormalities but are in a constant inflammatory state owing to the injections of contaminated materials. In addition, *S. aureus* itself can trigger inflammation and endothelial cell activation by secreting toxins like α-toxin, thereby facilitating its adhesion.



Take home figure Differential mechanisms of *S. aureus* adhesion to damaged or inflamed heart valves.

Both mechanisms are not mutually exclusive and probably overlap to some degree. In atherosclerotic and degenerative valve disease for example abnormal blood flow patterns create cardiac valve damage, but these conditions are also characterized by chronic inflammation.

As the validity of mouse models for human infectious diseases is sometimes disputed,[13] we first ascertained the validity of mice to study infective endocarditis by showing that similarly to humans, bacteraemic ageing mice also develop endocarditis. Nevertheless, older rodent models of endocarditis are inapt to study the early phase of endocarditis. In these models, a permanent transaortic catheter damages the valve of a mouse, rat, or rabbit, which then acts as a seeding ground for intravenously injected bacteria.[14] Although valuable and widely used, these models have several shortcomings. Firstly, they require the continuous presence of foreign material and therefore mimic a catheter infection rather than native valve endocarditis.[15] Secondly, they provide no information on the early phase of endocarditis and thirdly, they are solely based on cardiac valve damage and do not model inflammation-induced endocarditis.

Our new endocarditis model, although technically more challenging, was able to overcome these flaws. This model enables the study of early bacterial adhesion both in case of cardiac valve damage and inflammation and is the first rodent model in which real endocarditis occurs without the continuous presence of foreign material. We therefore believe that the model is highly relevant and able to provide more detailed information on the different stages of endocarditis compared with older models. A drawback of our model is, that to locally deliver histamine to the valves, the brief introduction of a transaortic catheter was indispensible. Bacterial adhesion in the inflammatory condition is therefore triggered by a combination of endothelium activation and limited cardiac valve damage. Nevertheless, the differences between the mechanisms that underlie adhesion and the detailed imaging of both conditions highlight that the inflammation- and damage-induced conditions truly represent distinct pathophysiological pathways.

Our findings might explain previous conflicting preclinical and clinical results concerning the antithrombotic treatment and prophylaxis of endocarditis.[3] In our model platelet depletion prevented bacterial adhesion in inflammation-induced endocarditis, but not in damage-induced valve infections, while the opposite holds true for fibrinogen depletion. In addition, these findings show that developing a vaccine to prevent *S. aureus* endocarditis might even be more complicated than thought. Most *S. aureus* vaccines are aimed against SrtA anchored adhesins, such as ClfA. Although these adhesins are involved in damaged-induced endocarditis, they appear obsolete in inflammation-induced endocarditis, in which bacteria seem to adhere in a non-SrtA mediated way to the aortic valve. Unfortunately, we were unable to identify the exact mechanism and further research is needed. In addition, because *S. aureus* Newman lacks functional fibronectin binding proteins no conclusions on their role in endocarditis could be made in this article.

In summary, using a new unique mouse model we were able to shine a light on some of the intricate mechanisms by which *S. aureus* is able to infect the heart valves under different conditions. These findings can provide inspiration for the development of much-needed new preventive strategies for endocarditis.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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