Molecular basis for Staphylococcus aureus-mediated platelet aggregate formation under arterial shear in vitro.

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Molecular basis for *Staphylococcus aureus*-mediated platelet aggregate formation under arterial shear *in vitro*

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SUMMARY

Staphylococcus aureus is the most frequent causative organism of infective endocarditis (IE) and is characterized by thrombus formation on a cardiac valve that can embolize to a distant site. Previously, we showed that *S. aureus* clumping factor A (ClfA) and fibronectin-binding protein A (FnBPA) can stimulate rapid platelet aggregation. In this study we investigate their relative roles in mediating aggregate formation under physiological shear conditions. Platelets failed to interact with immobilized wild-type *S. aureus* (Newman) at shear rates <500s^{-1} but rapidly formed an aggregate at shear rates >800s^{-1}. Inactivation of the ClfA gene eliminated aggregate formation at any shear rate. Using surrogate hosts that do not interact with platelets bacteria over-expressing ClfA supported rapid aggregate formation under high shear with a similar profile to Newman while bacteria over-expressing FnBPA did not. Fibrinogen binding to ClfA was found to be essential for aggregate formation although fibrinogen-coated surfaces only allowed single–platelets to adhere under all shear conditions. Blockade of the platelet immunoglobulin receptor FcγRIIa inhibited aggregate formation. Thus, fibrinogen and IgG binding to ClfA is essential for aggregate formation under arterial shear conditions and may explain why *S. aureus* is the major cause of IE.

Condensed abstract. Both *Staphylococcus aureus* clumping factor A (Clf A) and fibronectin-binding protein A (Fnbp A) mediate rapid platelet aggregation. However, only Clf A can induce rapid aggregate formation under arterial shear conditions that is dependent on antibody and fibrinogen binding. The corresponding platelet receptors are GPIIb/IIIa and FcγRIIa.

Infective endocarditis (IE) is characterized by the formation of platelet-bacteria thrombi on a heart valve which, if untreated, can lead to valve failure or the formation of infected emboli[1]. Infection of damaged or replacement valves is usually due to Streptococcus spp while infection of native valves is usually due to *Staphylococcus aureus* [2]. While many species of bacteria have been reported to cause endocarditis the majority of cases are due to *S. aureus* [3]. The reason for the dominance of *S. aureus*-mediated IE is not known.

The ability of bacteria to interact with platelets has been shown to be important in the pathogenesis of IE [1]. Bacteria can adhere to platelets and induce platelet aggregation. However these are two separate properties and are often mediated by distinct surface proteins[4]. Animal studies have shown that the ability of *S. aureus* to adhere to platelets and the ability of *S. sanguis* to induce platelet aggregation are important in infective endocarditis[5-8].

The complex interplay between platelets and bacteria is still not completely understood [9]. Successful colonization is likely to be the defining event leading to initiation of an infection[1]. Several different surface adhesins called microbial surface component reacting with adhesive matrix molecules (MSCRAMMs)[10] promote adhesion to and activation of platelets including clumping factor ClfA and fibronectin-binding proteins A and B (FnBPA and FnBPB)[12]. Both ClfA and FnBP bind fibrinogen allowing an interaction with platelet GPIIb/IIIa that supports bacterial adhesion to platelets. In the presence of specific antibodies to the MSCRAMM, engagement of the platelet Fc receptor FcγRIIa occurs which stimulates platelet activation and subsequent aggregation[11, 12].

There are several major concerns with the studies of *S. aureus*-platelet interactions. Firstly, most previous studies have been carried out under static (adhesion) or non-physiological stirring conditions (aggregation). Therefore, it is difficult to relate these studies to the disease process as cells in the vasculature experience a range of shear conditions. Some studies have been performed *in vitro* under shear to better characterize platelet-bacteria interactions under more physiological conditions. Studies using a cone and plate viscometer have shown that both protein A and ClfA are important in thrombus formation. However, extremely high shear levels were used[13]. A recent study showed a role for ClfA, SdrC, SdrD, SdrE and protein A in a parallel plate flow chamber[14] An earlier study showed a role for antibody in thrombus formation under shear [15].

Secondly, it may not be possible to investigate the interaction between *S. aureus* and platelets using an animal model. Several early studies suggest that platelets isolated from rabbits interact with *S. aureus* differently to platelets isolated from humans. For example, *S. aureus*-induced aggregation of rabbit platelets was biphasic and was not inhibited by an RGD peptide that binds to GPIIb/IIIa[16,17]. This is in direct contrast to *S. aureus*-induced aggregation of human platelets which is monophasic and is inhibited by RGD peptides. Moreover, several studies have now highlighted the importance of the platelet Fc receptor, FcγRIIa, in platelet activation by *S. aureus* [11,12]. Murine platelets do not express FcγRIIa[18], suggesting that the mechanism of *S. aureus* induced platelet aggregation differs between human platelet and murine platelets.

To address this problem we investigated the molecular mechanisms of *S. aureus*-induced human platelet aggregate formation under venous and arterial shear, using a parallel flow chamber. We also investigate the relative roles of clumping factor A and fibronectin-binding proteins A in the interaction between platelets and immobilized *S. aureus* in a fluid shear environment and assess the role of plasma proteins in aggregate formation under both arterial and venous shear rates. We show that ClfA is expressed on the bacterial cell surface and mediates rapid aggregate formation under high shear in a process that requires both fibrinogen and anti-ClfA antibodies, while FnBPA does not play a role in thrombus formation.

Methods

Materials

Plasminogen-depleted human plasma fibrinogen and bovine serum albumin (BSA) were purchased from Calbiochem, Nottingham, UK. Purified pooled human IgG was obtained from Baxter, UK. Platelet $\alpha_{IIb}\beta_3$ antagonist, tirofiban, was purchased from Merck, UK. Platelet anti-FcγRIIa antibody, IV.3 was a kind gift from Dr. J Kimkowsky, Medarex Inc. NJ, USA. Lipophilic dye, 3,3'-dihexyloxacarbocyanine iodide (DiOC6) was purchased from Biosciences, Franklin Lakes, NJ, USA. Flow chambers were purchased from GlycoTech, Rockville, MD, USA. Glass cover slides were purchased from BioWorld, Atlanta, GA, USA. Bacterial growth media were purchased from Oxoid Ltd, Basingstoke, UK. All other reagents were purchased from Sigma, Poole, UK

Bacterial strains and growth conditions

S. aureus was grown in brain heart infusion (BHI) broth at 37ºC with shaking (200rpm) for 18hrs (stationary phase). *S. aureus* Newman defective in clumping factor A has been described elsewhere[19]. *Lactococcus lactis* was used to carry empty vector (pKS80) or for heterologous expression of the *S. aureus* surface protein ClfA (pKS80clfA+)[20]. *L. lactis* was routinely grown in M17 agar or statically in M17 broth incorporating 0.5% (w/v) glucose at 30ºC for 18 hrs[21]. *S. aureus* strain 8325-4 which lacks FnBPA and the plasmid containing the entire fnBPA gene have been described elsewhere[22]. Antibiotics were incorporated into the growth media where appropriate: erythromycin (5µg/ml) and ampicillin (100µg/ml). Bacteria were harvested and washed twice by centrifugation at 15,000xg for 5 minutes. Washed bacteria were finally resuspended in phosphate buffered saline (PBS) and adjusted to an optical density of 1.4.

Platelet preparation

Peripheral blood was collected from healthy human adult volunteers after informed consent. Nine volumes of whole blood were collected into one volume of 3.8% sodium citrate. Platelet-rich plasma (PRP) was prepared as previously described[4]. To prepare platelets free of plasma proteins, prostaglandin E_1 (1µM) and apyrase (1U/ml) were added to PRP and centrifuged at 650xg for 10 minutes. The plasma was removed from the platelet pellet and resuspended in the same volume of JNL buffer (6mM dextrose, 130mM NaCl, 9mM $NaCl_2$, 10mM Na citrate, 10mM Tris base, 3mM KCL, 0.8 KH_2PO_4 and 0.9mM $MgCl_2$). These plasma-free platelets were resuspended in physiological buffer were added back to whole blood for some experiments.

Preparation of flow chamber slides.

A 1ml solution of either fibrinogen (20µg/ml) or *S. aureus* (10^9 bacterial cells) was applied to glass slides (75 x 25 mm) and allowed to attach overnight at 4ºC or for 2hrs at room temperature in a humidity chamber. Uniform bacterial coverage on glass slides was verified by crystal violet staining. Slides were washed 3 times in PBS buffer to remove any unbound bacteria or fibrinogen. Finally, the slides were blocked with 1% BSA for a further 1 hour at 37ºC.

L. lactis failed to adhere to the glass slides (probably due to its surface charge) therefore it was necessary to generate appropriate charges on both the glass slides and bacterial surface. Glass slides were submersed in 1% Triton-X 100 for 30 minutes and washed in running tap water for a further 30 minutes. The slides were then sterilized using 95% EtOH and allowed air dry. To produce a covalently bound amino group on the glass surface the slides were submersed in freshly prepared 2% solution of 3-aminopropyltriethoxysilane in dry acetone for 5 seconds. Finally, glass slides were

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washed in distilled water (5 minutes x 2) and dried overnight at 42ºC. This method produces a surface covered in amino groups which will covalently bind to free aldehydes. Bacterial cells were centrifuged at 15,000xg. Pellets were resuspended in 5mls 0.5% formaldehyde for 1hour at room temperature with occasional mixing. The suspension of bacterial cells was finally centrifuged at 15,000xg and the resultant pellet resuspended and adjusted to a final OD of 1.4 using PBS. This method creates a bacterial surface covered in free aldehydes capable of covalently binding to the amino groups on the silane treated slides.

Epi-fluorescence digital microscopy

Platelet interaction with immobilized protein or bacteria under various flow conditions was studied using a parallel flow chamber. Platelets were labeled in whole blood by direct incubation with the fluorescent lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6). A syringe pump (Harvard Biosciences, MA, USA) was used to aspirate blood through the flow chamber. Platelet adhesion and aggregate formation was visualized using phase contrast and fluorescent imaging microscopy (63X oil immersion lens-Achroplan objective) through the flow chamber (GlycoTech, Rockville, MD, US) mounted on a Zeiss Axiovert-200 epi-fluorescence microscope (Carl Zeiss, Welwyn Garden City, UK). Images were taken at the same downstream location (from flow chamber entrance) in all experiments. Images were captured every second up to 300s by a liquid chilled Quantix-57 CCD camera (Photometrics Ltd, Tuscon, AZ, USA). Platelet adhesion and aggregate formation was analyzed using MetaMorph software (Universal Imaging Corp., Downingtown, PA, USA).

Statistical Analysis

Statistics were performed using InStat statistical software (GraphPad software, SD, USA). Data shown are the means plus or minus standard error of the mean (SEM) and comparisons between mean values were performed using the Student paired or unpaired *t*-test.

RESULTS

Aggregate formation on immobilized S. aureus *strain Newman under shear conditions*

Using bright-field-imaging microscopy platelets (visualized as black cells) perfused under low shear conditions ($50s^{-1}$) in plasma failed to interact with immobilized *S. aureus* Newman after 300 seconds (fig 1a). At higher shear rates ($>100s^{-1}$) cells are concentrated in the centre of the flow stream making interaction with the surface difficult, however, if whole blood is used the larger cells (red and white blood cells) are carried to the centre of the main stream of flow, leaving the smaller cells (platelets) in a better position to interact with immobilized bacteria on the glass slides. Platelets in whole blood stained with a lipophilic dye also failed to interact with immobilized *S. aureus* Newman when the shear rate was increased to $200s^{-1}$ or $500s^{-1}$ (data not shown).

Increasing the shear rate to $800s^{-1}$ led to adhesion of platelets to immobilized *S. aureus* strain Newman. Platelet aggregates began to form (white areas) within 100 seconds and became progressively larger as perfusion continued (fig 1b). After 300 seconds aggregate formation covered almost the entire visible field of view (1±0.2% coverage at $50s^{-1}$ and 90±5% coverage at $800s^{-1}$, n=3, $P<0.0001$). Perfusing whole blood at $1500s^{-1}$ led to more rapid platelet deposition and aggregate formation than at $800s^{-1}$. Large platelet aggregates began to form at 100 seconds leading to complete occlusion of the field of view by 200 seconds. After 250 seconds the aggregate fragmented leaving behind the immobilized *S. aureus* Newman. With further perfusion of

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platelets the process of aggregate formation began again on the remaining immobilized *S. aureus* Newman (fig 1b). Platelets perfused at $800s^{-1}$ failed to interact with *S. aureus* strain 8325-4 (fig 1c, 90±5% coverage on Newman and 0±0.0% coverage on 8325-4, n=3, $P<0.0001$).

Aggregate formation on immobilized S. aureus *strain Newman lacking ClfA expression*

The ability of the ClfA-containing strain Newman to induce platelet aggregation is in contrast to the lack of activity of strain 8325-4 which does not contain ClfA and suggests a role for ClfA in the interaction with platelets. It also suggests that this protein may play a more dominant role in aggregate formation than FnBPA. To address this question we studied a ClfA mutant of *S. aureus* Newman ($ClfA^-$). Platelets in whole blood perfused at $800s^{-1}$ failed to interact with Newman $ClfA^-$ after 300 seconds (fig 2b; 90±5% coverage on Newman $ClfA^+$ and 2±1% coverage on Newman $ClfA^-$, n=3, $P<0.0001$) or at the higher shear rate of $1500s^{-1}$ (data not shown).

Aggregate formation on immobilized surrogate hosts over expressing ClfA or FnBPA

To confirm the relative importance of ClfA in mediating aggregate formation under high shear both ClfA and FnBP A were separately over-expressed in surrogate hosts. Platelets perfused in whole blood failed to interact with fixed *L. lactis* at any shear rate tested (fig 3a). However, platelets perfused at $800s^{-1}$ time-dependently adhered to fixed *L. lactis* expressing ClfA. After 100 seconds large platelet aggregates formed with eventual complete occlusion at 300 seconds (fig 3b) in a similar fashion to *S. aureus* Newman (0.5±0.1% coverage on *L. lactis* pKS80 and 89±4% coverage on *L. lactis* $ClfA^+$, n=3, $P<0.0001$,). In contrast, platelets perfused in whole blood at $800s^{-1}$ failed to interact with either *S. aureus* strain 8325-4 (fig 3c) or *S. aureus* 8325-4 over expressing FnBPA (0±0.0% coverage on 8325-4 $FnBPA^-$ and 1±0.2% coverage on $FnBP^+$, n=3, NS). Comparative analysis by flow cytometry and western blot of *S. aureus* Newman and *L. lactis* cells expressing ClfA showed that *L. lactis* expressed similar levels of protein on their surface compared to *S. aureus*. Furthermore, western blot data demonstrated high levels of FnBPA in *S. aureus* strain 8325-4 (data not shown).

Platelet adhesion to immobilized fibrinogen at high and low shear

We next investigated the molecular mechanisms following ClfA-induced aggregate formation. Fibrinogen plays a major role in platelet aggregate formation, an important step in thrombus formation. To investigate if the platelet interaction with *S. aureus* was simply due to ClfA binding fibrinogen and presenting passing platelets with a thrombogenic surface, we immobilized purified fibrinogen and perfused platelets at both high and low shear. Under low shear conditions ($150s^{-1}$) single platelets adhered to the immobilized fibrinogen in a time-dependent manner (fig 4a). Increasing the shear rate to $800s^{-1}$ also led to the development of a monolayer of single platelets but no aggregate was formed even at 300 seconds (fig 4b). These results suggest that fibrinogen binding to ClfA is not sufficient to induce aggregate formation alone and that other plasma factors may play a role.

Platelet activation following L. lactis *ClfA - induced aggregate formation.*

To dissect the mechanisms leading to aggregate formation, whole blood was treated with aspirin (10μM) and perfused over immobilized *L. lactis* ClfA at $800s^{-1}$. Aggregate formation was strongly inhibited but single platelet adhesion occurred (fig 5c; 89±4% coverage with *L. lactis* ClfA and 7±1% coverage when treated with aspirin, n=3, $P<0.0001$). We

next assessed the importance of fibrinogen binding to platelets in initiating aggregate formation. Addition of the GPIIb/IIIa antagonist, tirofiban (0.5μM) to blood completely inhibited both aggregate formation and single platelet adhesion (fig 5d; 89±4% coverage over *L. lactis* ClfA and 4±0.1% coverage in the presence of tirofiban, n=3, *P*<0.0001). These results suggest that GPIIb/IIIa and cyclooxygenase play an essential role in aggregate formation in this model system.

Role of plasma proteins in S. aureus *ClfA-induced aggregate formation.*

As platelets perfused under high shear ($800s^{-1}$) are capable of interacting with immobilized *L. lactis* ClfA we set out to determine if plasma factors are involved in aggregate formation. Single platelet adhesion was observed when gel-filtered platelets were combined with washed red blood cells and perfused over immobilized *L. lactis* ClfA (fig 6a; 13±2% coverage in plasma-free platelets perfused over *L. lactis* ClfA and 89±4% coverage when plasma was included, n=3, P<0.001). Single platelet adhesion was also observed when antibody purified fibrinogen was also added (fig 6b, 89±4% coverage with plasma-rich platelets perfused over *L. lactis* ClfA and 9±0.2% coverage with plasma-free platelets supplemented with fibrinogen, n=3, *P*<0.002). No interaction was observed when pooled human IgG was added (fig 6c; 89±4% coverage with plasma-rich platelets perfused over *L. lactis* ClfA and 2±0.5% coverage with plasma-free platelets supplemented with IgG, n=3, *P*<0.0001). However, when washed blood cells were combined with fibrinogen and pooled human IgG time-dependent aggregate formation (fig 6d) similar to that with whole blood was observed (89±4% coverage with plasma-rich platelets perfused over *L. lactis* ClfA and 74±3% coverage with plasma-free platelets supplemented with fibrinogen and IgG, n=3, *P*=NS). When gel-filtered platelets were treated with the monoclonal antibody IV-3 aggregate formation was reduced to single platelet adhesion (fig 6e; 89±4% coverage with plasma-rich platelets perfused over *L. lactis* ClfA and 8±2% coverage in the presence of IV.3, n=3, *P*<0.002).

CONCLUSIONS

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S. aureus is the major cause of infective endocarditis and its ability to interact with platelets is essential for infection of heart valves[23]. Previous studies have shown that *S. aureus* expresses a number of proteins capable of activating platelets including ClfA, FnBPA, and FnBPB[21],[12]. However the relative importance of the individual proteins is not clear. This is complicated by the fact that the roles of these proteins have been characterized with *in vitro* assays such as platelet aggregation which may not reflect the situation *in vivo*.

Using a parallel plate chamber and a range of shear rates we investigated the ability of platelets to interact with immobilized *S. aureus*. The *S. aureus* strain Newman expressing ClfA induces platelet aggregation with a short lag time and strongly supports platelet adhesion under static conditions in the presence of fibrinogen. Under low shear conditions (venous shear) there is no evidence of platelet adhesion, presumably due to the weakness of the interaction which cannot support platelet attachment under shear stress. However, under high shear conditions (arterial shear) very strong adhesion occurred followed by rapid aggregate formation. This is an unique interaction in that other IE microorganisms such as *S. sanguis*[24] or *S.* gordonii[25] failed to interact at high shear however did interact at low shear. The aggregate formation is not solely due to platelets attaching to fibrinogen bound to the surface of immobilized bacteria as immobilized fibrinogen only supported single platelet adhesion without aggregate formation. This phenomenon of only

supporting platelet adhesion under high shear and not low shear is similar to that seen with vWf however; vWf is not involved in the interaction described here. Fibrinogen bound to ClfA will be subject to shear stress under flow conditions. The effect of shear will be to alter the conformation of fibrinogen. This activated conformation may be more effective at interacting with GPIIb/IIIa and is more likely to occur under high shear. So, under low shear bound fibrinogen can still interact with GPIIb/IIIa but this interaction is not strong enough to resist the shear stress preventing thrombus formation but at high shear the strength of the interaction is increased resulting in aggregate formation

To characterize this interaction further, we examined the role of MSCRAMMs, the major platelet-interacting proteins of *S. aureus*. A ClfA defective mutant (ClfA$^-$) of *S. aureus* Newman could still induce platelet aggregation (albeit with a longer lag time) and support platelet adhesion (data not shown). However, the mutant failed to support strong adhesion or aggregate formation under any shear condition. The essential role of ClfA in the interaction was confirmed by expression in the surrogate host *L. lactis*. *L. lactis* ClfA$^+$ was as effective as Newman at inducing aggregation and adhesion under shear (data not shown). Thus, while there are a number of surface proteins on Newman capable of activating platelets, the only one that can trigger aggregate formation under shear is ClfA.

Pawar *et al* demonstrated that protein A has the capability of binding to vWf under high shear but not low shear conditions[13]. This interaction acts as a bridging mechanism for crosslinking to platelet GPIb thereby initiating platelet adhesion. These experiments were carried out with *S. aureus* cells in suspension mixed with platelet-rich plasma and subjected to shear rates only found in severe pathological conditions ($5000s^{-1}$). A more recent study demonstrated that *L. lactis* expressing protein A adhered time-dependently to immobilized von Willebrand factor at low shear rates ($100s^{-1}$)[26]. Together these results suggest that the protein A – von Willebrand factor interaction may mediate the initial interaction leading to immobilization of bacteria at the site of injury but it is unlikely that protein A alone causes thrombus formation. We have shown that platelets fail to interact with immobilized *L. lactis* expressing protein A at any shear rate[26] so it is more likely to act as a co-stimulator rather than a primary activator.

Previous studies have shown that ClfA binds to fibrinogen which in turn binds to the platelet integrin GPIIb/IIIa. However, binding fibrinogen per se is not enough to induce platelet aggregation or lead to aggregate generation. When platelets were perfused over immobilized fibrinogen at either high or low shear, single platelet adhesion was observed but not aggregate formation. *S. aureus* is a common commensal of the human body and antibodies to surface proteins are present in the plasma of most if not all healthy individuals. We have shown previously that specific anti-ClfA antibodies are required for ClfA-induced platelet aggregation[11] therefore we investigated their role in platelet interactions with bacteria under shear stress. Antibody binding to ClfA is itself not sufficient to induce aggregate formation. However, when fibrinogen and specific antibody together bound to ClfA, aggregate formation occurred. The role of antibody is to interact with the platelet Fc receptor FcγRIIa. A function-blocking monoclonal antibody directed against FcγRIIa inhibited the rapid aggregate formation. A requirement for antibody is in agreement with the previous study of Sjobring and co-workers [15].

Several studies have recently identified the binding capabilities of *S. aureus* FnBP's to human blood platelets[12,27]. Our results presented here do not disagree with these findings but have demonstrated that under high shear conditions FnBP's do not have the ability to interact with or induce a aggregate formation. Similarly recent studies have suggested that FnBP's play a role in binding to healthy resting endothelial cells[28], however under shear this interaction does not take place[29]. Collectively these results suggest that the fibronectin-binding protein may have little or no role in aggregate formation but as IE is a complex disease they may still play a role in the pathology of the disease. It is also important to note that while there is high shear in the coronary vessels the situation is much more complex at the valve surface where turbulence is also likely to play a role.

In summary the results from this study demonstrate, that the simultaneous binding of fibrinogen and antibody to ClfA, which interact with GPIIb/IIIa and FcγRIIa on the platelet surface, is essential for aggregate formation. This rapid aggregate formation is unique to ClfA and may explain the high incidence of *S. aureus* in infective endocarditis as it is a very powerful trigger of aggregate formation in the presence of anti-*S. aureus* antibody. ClfA may be a good candidate for a vaccine for patients at high risk of IE. However, it is essential that the antibodies generated would block the ClfA-fibrinogen interaction otherwise the presence of high levels of non-neutralizing antibody would likely exacerbate IE.

FIGURE LEGENDS

Figure 1. Platelet adhesion to immobilized *S. aureus* Newman under low and high shear conditions. **A.** *S. aureus* Newman was coated onto glass cover slips for 2 hours at room temperature. Platelet-rich plasma was perfused over immobilized *S. aureus* Newman at $50s^{-1}$. For higher shear experiments platelets were labeled in whole blood by direct incubation with a fluorescent lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6). Whole blood was perfused at $800\,s^{-1}$ over immobilized *S. aureus* Newman (A) or 8325-4 (B). Images were captured every second up to 300s. Images are representative fields taken from 1 of 3 independent experiments that yielded similar results.

Figure 2. Aggregate formation on immobilized *S. aureus* Newman. *S. aureus* Newman (A) and *S. aureus* Newman lacking expression of ClfA (ClfA$^-$) (B) were coated onto glass cover slips for 2 hours at room temperature. Platelets were labeled in whole blood by incubation with a fluorescent lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6). Whole blood was perfused over immobilized bacteria at $800\,s^{-1}$. Images were captured every second up to 300s. Images are representative fields taken from 1 of 3 independent experiments that yielded similar results.

Figure 3. Rapid aggregate formation on immobilized surrogate hosts over-expressing ClfA and FnBPA under high shear conditions. *L. lactis* (A), *L. lactis* ClfA (B), *S. aureus* 8325-4 (C) and *S. aureus* 8325-4 FnBPA$^+$ (D) were coated onto glass coverslips for 2 hours at room temperature. Platelets were labeled in whole blood by incubation with a fluorescent lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6). Whole blood was perfused over bacteria at $800\,s^{-1}$. Images were captured every second up to 300s. Images are representative fields taken from 1 of 3 independent experiments that yielded similar results.

Figure 4. Platelet adhesion to immobilized fibrinogen. Fibrinogen (20µg/ml) was coated on glass cover slips for 2 hours at room temperature. Platelets were labeled in

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whole blood by incubation with a fluorescent lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6) and perfused over immobilized fibrinogen at low ($150s^{-1}$) and high ($800s^{-1}$) shear. Platelet adhesion was visualized using epifluorescent digital imaging microscopy. Images were captured every second up to 300s. Images are representative fields taken from 1 of 3 independent experiments that yielded similar results.

Figure 5. Inhibition of ClfA-induced aggregate formation. *L. lactis* (A) and *L. lactis* ClfA (B, C & D) were coated onto glass coverslips for 2 hours at room temperature. Platelets were labeled in whole blood by incubation with a fluorescent lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6). Whole blood was treated with aspirin (C) or tirofiban (D) for 20 minutes. Drug treated whole blood was perfused over bacteria at $800\,s^{-1}$. Images were captured every second up to 300s. Images are representative fields taken from 1 of 3 independent experiments that yielded similar results.

Figure 6. The role of plasma proteins in *L. lactis* ClfA- induced aggregate formation. *L. lactis* (A) and *L. lactis* ClfA (B, C, D, E) were coated onto glass coverslips for 2 hours at room temperature. Platelets were separated from whole blood and plasma by centrifugation. Platelets were resuspended in physiological buffer and added back to whole blood. Platelets were labeled in whole blood by incubation with a fluorescent lipophilic dye 3,3'-dihexyloxacarbocyanine iodide (DiOC6). Whole blood supplemented with either fibrinogen only (B), IgG only (C) or a combination of fibrinogen and IgG in the absence of (D) and presence of (E) the FcγRIIa inhibitor IV.3 was perfused over bacteria at $800\,s^{-1}$. Images were captured every second up to 300s. Images are representative fields taken from 1 of 3 independent experiments that yielded similar results.

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FIGURE 1

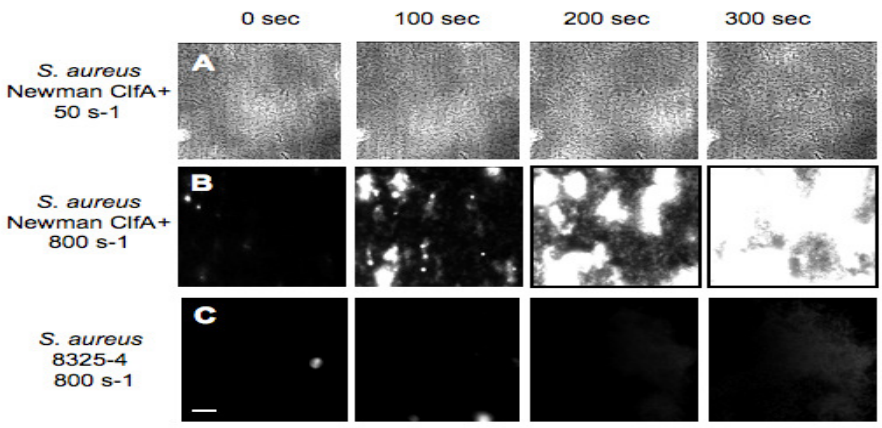

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FIGURE 2

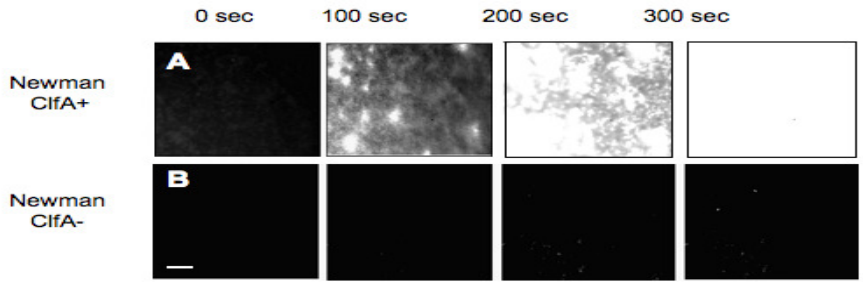


FIGURE 3

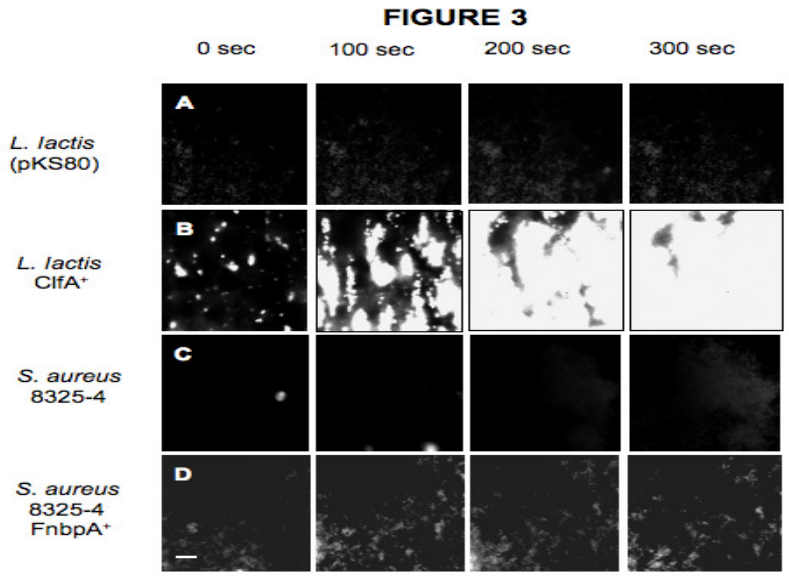

0 sec
100 sec
200 sec
300 sec
A
L. lactis (pKS80)
B
L. lactis ClfA+
C
S. aureus 8325-4
D
S. aureus 8325-4 FnbpA+

FIGURE 4

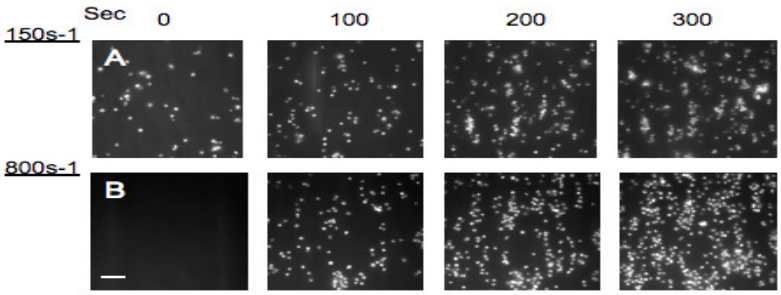


FIGURE 5

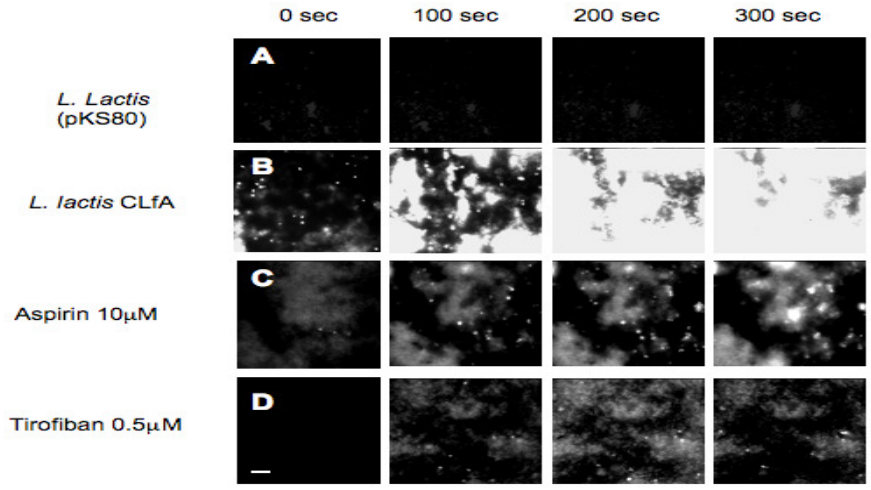
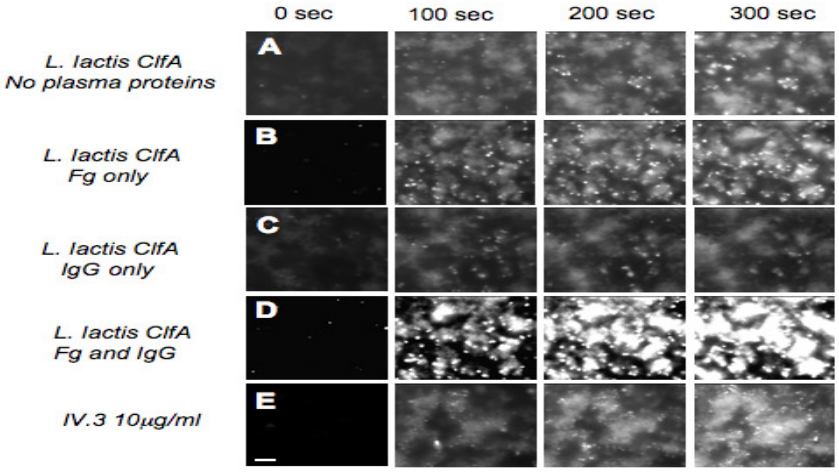

0 sec
100 sec
200 sec
300 sec
A
B
C
D
L. Lactis (pKS80)
L. lactis CLfA
Aspirin 10μM
Tirofiban 0.5μM

FIGURE 6


0 sec
100 sec
200 sec
300 sec
A
B
C
D
E
L. lactis ClfA
No plasma proteins
L. lactis ClfA
Fg only
L. lactis ClfA
IgG only
L. lactis ClfA
Fg and IgG
IV.3 10μg/ml