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***Staphylococcus aureus* lipoteichoic acid inhibits platelet activation and thrombus formation via the Paf receptor**

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## Abstract

Impaired healing is common in wounds infected with the major human pathogen *Staphylococcus aureus*, although the underlying mechanisms are poorly understood. Here, we show that *S.aureus* lipoteichoic acid (LTA) inhibits platelet aggregation caused by physiological agonists and *S. aureus* and reduced platelet thrombus formation *in vitro*. The presence of D-alanine on LTA is necessary for the full inhibitory effect. Inhibition of aggregation was blocked using a monoclonal anti-platelet activating factor receptor (PafR) antibody and Ginkgolide B, a well-defined PafR antagonist, demonstrating that the LTA inhibitory signal occurs via PafR. Using a cyclic AMP (cAMP) assay and a western blot for phosphorylated VASP, we determined that cAMP levels increase upon platelet incubation with LTA, an effect which inhibits platelet activation. This was blocked when platelets were preincubated with Ginkgolide B. Furthermore, LTA reduced haemostasis in a mouse tail-bleed assay.

## Introduction

*Staphylococcus aureus* is an important opportunistic human pathogen and the cause of a large burden of morbidity and mortality. The ability of the pathogen to bind to and activate platelets, small (2-4 $\mu$ m), blood cells responsible for maintaining normal haemostasis, leads to the formation of platelet-bacteria thrombi on the surface of heart valves, which is required for the development of endocarditis since platelets attached to damaged valves serve as foci for attachment of bacteria circulating in the blood[1]. Several studies have shown that *S. aureus* binds platelets and induces their aggregation. The pathogen possesses a variety of surface proteins known as microbial surface components reacting with adhesive matrix molecules (MSCRAMMs), some of which are virulence factors in models of *S. aureus* endocarditis. MSCRAMMs attach the bacterium to platelets, either indirectly, by binding to fibrinogen, simultaneously bound to the platelet surface by integrin  $\alpha$ IIb $\beta$ 3, or by binding directly to  $\alpha$ IIb $\beta$ 3, thus inducing outside-in signalling and platelet activation[2,3]\_ENREF\_8\_ENREF\_13. Such observations rely on washed *S. aureus* cells and thus ignore the contribution of bacterial molecules secreted into the extracellular milieu.

Although induction of thrombus formation by *S. aureus* has been characterised extensively, infection of wounds by this pathogen frequently results in impaired healing; the mechanisms of which are not fully understood[4]. *S. aureus* extracellular proteins Efb inhibits platelet aggregation by binding to fibrinogen[5]\_ENREF\_10. Inhibition of platelet activity by Efb or pharmacological

antagonists causes decreased killing of *S. aureus* in whole blood and increases the lethality of *S. aureus* infection in a mouse model[6].

*S. aureus* lipoteichoic acid (LTA) was previously shown to inhibit activation of platelets, although a role in haemostasis, its relevance to the *S. aureus*-platelet interaction and the mechanism(s) by which inhibition is achieved are not understood[7]. In this study, LTA inhibited activation of human platelets by physiological agonists and *S. aureus*. Furthermore LTA inhibits platelet function and thrombus formation *in vivo* by binding platelet activating factor receptor (PafR), a phospholipid receptor that binds LTA and is associated with an increased platelet intracellular cyclic AMP (cAMP) concentration.

## Methods

### Reagents

Collagen was obtained from Nycomed, thrombin, Ginkgolide B, Mouse IgG from Sigma and cross-linked collagen-related peptide (CRP-XL) from R. Farndale (University of Cambridge, UK). Anti-LTA (pagibaximab) was provided by Biosynexus Inc. Anti-TLR2 (T2.5) was purchased from eBioscience, anti-CD14 (UCH-M1) from AbD serotec, anti-PafR from Cayman Chemical, anti-PhosphoVASP from Cell Signaling Technology.

### Bacterial strains used

Wild type *S. aureus* SA113 was used with *S. aureus* SA113  $\Delta dltABCD$ [8], *S. aureus* SA113  $\Delta tagO$ [9] and *S. aureus* SA113  $\Delta lgt$ [10]. *S. aureus* SEJ1 and

isogenic strains  $\Delta gdpP$ ,  $\Delta gdpP\Delta ltaS$  and pCN34-*ltaS* were used for mutant studies[11]. *Bacillus subtilis* 128 and *Streptococcus pneumoniae* D34 were used.

### **LTA extraction**

*S. aureus* was grown in BHI 37°C and centrifuged at 20463g for 15min. The pellet was re-suspended in 50% butanol/water. LTA was re-suspended in a 1mM sodium acetate, 15% 1-propanol buffer followed by a 15-60% 1-propanol elution gradient, dialysed against dH<sub>2</sub>O and the concentration determined by phosphate assay[12].

### **LTA from *S. aureus* culture supernatants**

*S. aureus* cultures were centrifuged at *c.* 12,000g for 10min to remove cells. 2.3M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the supernatant overnight at 4°C. The supernatant was centrifuged at 20,000g for 20min at 4°C and the pellet resuspended in 2mL of PBS. To standardise supernatant preparations, including those lacking LTA, proteins carried over were quantified by Bradford assay. Where appropriate, LTA concentrations were determined as above.

### **Preparation of human platelets**

Human blood was obtained from healthy volunteers who gave informed consent. Ethical approval was obtained from the University of Reading Research Ethics Committee. Platelets were prepared as described previously[13].  $4 \times 10^8$  platelets/mL were incubated for 15min with LTA and stimulated by agonists. Aggregation was measured in an optical aggregometer (Chronolog). Percentage inhibition of aggregation by LTA was calculated by dividing maximal aggregation

of LTA treated samples by the aggregation achieved by the given agonist alone. Centrifuged *S. aureus* were washed three times in Tyrodes buffer and adjusted for a final experimental OD<sub>600</sub> 0.3. Aggregation was measured up to 15mins.

### **Flow cytometry**

5µL of PRP (4 x 10<sup>8</sup> cells/mL), was incubated with anti-PafR (50µg/mL), IgG2a (50µg/mL) or Tyrode's buffer for 30mins, then incubated with various concentrations of FITC-LTA for 15mins. Fluorescence intensity of the sample was measured using a BD Accuri™ C6 flow cytometry. 10,000 events per sample were measured.

### **Measurement of intracellular [Ca<sup>2+</sup>]<sub>i</sub>**

Platelets were pre-loaded with the fluorescent dye Fluo-4NW as described previously[13]. PRP was pre-incubated with LTA for 15min before being stimulated with CRP-XL, calcium release was measured using a Fluoroskan reader (Thermolab Systems) at 485/530nm.

### ***In vitro* thrombus formation**

Whole citrated blood was perfused through a Vena8™Biochip (Cellex, Dublin). Z-stack images were taken every 30s using a Nikon eclipse (TE2000-U) microscope data was analyzed using Slidebook™5 software (Intelligent Imaging Innovations, Denver, USA)

### **Tail-bleeding assay**

Procedures were approved by the University of Reading Animal Ethics Committee and the Home Office. Sixteen age matched C57BL/6 mice were anesthetized using ketamine (80mg/kg) and xylazine (5mg/kg) administered intra-peritoneally prior to a tail biopsy. Time to cessation of bleeding was measured up to 20min.

### **Results**

#### ***S. aureus* LTA inhibits platelets aggregation by various platelet agonists**

LTA is found at high concentrations within *S. aureus* cells and the extracellular milieu[14] and could thus interact with platelets during infection. LTA present in the supernatant and *S. aureus* cell envelope have the same structure[15]. In this study we investigated LTA inhibition of platelet aggregation. Firstly, we confirmed equal inhibitory activity for LTA purified from the supernatant and cells (Fig. S1A). For efficiency, remaining experiments used LTA extracted from the cell envelope. To determine which signaling pathways LTA inhibits, aggregation assays were performed with well characterized platelet agonists. Purified LTA from *S. aureus* SA113 was pre-incubated with washed human platelets before activation with CRP-XL, a collagen receptor Glycoprotein VI selective agonist (Fig. 1Ai, Aii), platelet activating factor (paf) (Fig. 1Bi, Bii) or thrombin (Fig. 1Ci, Cii). LTA inhibited aggregation in a dose dependent manner with all agonists. LTA was incubated for varying periods of time with washed platelets to observe any time dependent effects on aggregation. Using 4µg/mL LTA, inhibition of aggregation increased in a time dependent manner (Fig. 1Di, Dii). Platelet activation was inhibited over extended time periods (Fig. S1B). The highest concentration of LTA used with thrombin (3µg/mL), CRP-XL (4µg/mL)

and paf (16µg/mL) as platelet agonists produced a 40%, 85% and 50% reduction in platelet aggregation respectively, thus showing LTA to be a potent inhibitor of platelet aggregation.

Whole bacterial cells stimulate platelet activation via formation of fibrinogen or fibronectin bridges between integrin  $\alpha$ IIb $\beta$ 3 and *S. aureus* MSCRAMMs[3,16,17]. Having demonstrated LTA inhibition of platelet activation by physiological agonists, we examined the ability of exogenous LTA to inhibit *S. aureus* induced platelet aggregation in PRP (Fig. 1E). Increasing LTA concentrations increased the lag time to platelet aggregation.

To confirm that the observed inhibition was due to LTA rather than a co-purifying contaminant, platelets were pre-treated with monoclonal anti-LTA and LTA before stimulation with CRP-XL. This blocked the LTA inhibitory effect (Figs. 2Ai, 2Aii). An isotype (IgG1) matched control had no effect (results not shown). Lipoproteins that can co-purify with LTA are sometimes responsible for the immunological activities that have been assigned to LTA[18]\_ENREF\_36. *S. aureus* wall teichoic acid (WTA) has a similar structure to LTA. LTA was extracted from SA113  $\Delta$ tagO and SA113  $\Delta$ lgt, which lack WTA and lipoproteins respectively, and tested in the same manner. LTA extracted from both of these strains inhibited platelets to the same levels as LTA from SA113 (Fig. S2), confirming LTA platelet inhibitory activity and excluding any effect from lipoproteins or WTA.

To determine whether this inhibition was restricted to *S. aureus* LTA, the molecule was extracted and purified from two other Gram positive bacteria and tested for its ability to inhibit platelet aggregation. *Bacillus subtilis* is a non-pathogenic species which, like *S. aureus*, produces LTA with a 1,3-linked polyglycerolphosphate chain tethered to the membrane by a diglucosyl-diacylglycerol glycolipid. Glycerolphosphate subunits are esterified with d-alanine[19]. *Streptococcus pneumoniae* LTA consists of a repeating ribitol-galactose backbone with phosphocholine and D-alanine residues attached[20]. *S. pneumoniae* LTA showed no inhibition, whereas *B. subtilis* LTA caused a dose-dependent inhibition up to a maximum of 20% (Fig. 2Bi, 2Bii, 2C); we were unable to solubilise high enough concentrations of *B. subtilis* LTA to achieve saturation.

#### **The supernatant of an *S. aureus* mutant which lacks LTA is unable to inhibit platelet activation**

*S. aureus* mutant strains lacking LTA only grow under osmotically stabilising conditions or by acquiring compensatory mutations[11]. *S. aureus* SEJ1 (RN4220 *spa*) was used to construct an LTA-deficient ( $\Delta$ *ltaS*) strain, but in order for the  $\Delta$ *ltaS* to be viable, *gdpP* must also be mutated[11]. Cells of the parental SEJ1 and isogenic strains  $\Delta$ *gdpP*,  $\Delta$ *gdpP* $\Delta$ *ltaS* and  $\Delta$ *gdpP* $\Delta$ *ltaS* containing pCN34-*ltaS*, a complementation plasmid expressing *ltaS* from its native promoter, were grown and OD<sub>600</sub> of c. 0.5 and LTA in the supernatant was precipitated using (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. To ensure that consistent amounts of material were used, the amount of protein precipitated along with the LTA was determined by Bradford assay to standardise the preparations. As expected, only supernatant from the  $\Delta$ *gdpP* $\Delta$ *ltaS* lacked LTA

upon Western blotting (results not shown). In each experiment, precipitate was used to a final concentration of 10µg/mL of exoprotein. Supernatant from all strains, except *ΔgdpPΔltaS*, inhibited platelet activation (Fig. 2Di, Dii).

We were unable to assess the ability of these strains to induce platelet aggregation, as *S. aureus* RN4220 proved unable to induce aggregation despite incubations of up to 1 hour (results not shown).

### **LTA produced from *S. aureus* *ΔdltABCD* has a reduced ability to inhibit platelet aggregation**

D-alanine residues are important for various functions of LTA in different biological systems. In *S. aureus* the addition of D-alanine to the LTA chain is encoded by the *dlt* operon[8]. LTA was extracted and purified from *S. aureus* SA113 *ΔdltABCD* and pre-incubated with washed human platelets. LTA purified from *S. aureus* SA113 *ΔdltABCD* showed a significant reduction (*c.* 60%) in its ability to inhibit the platelet aggregation (Fig. 3Ai), compared to LTA from the parental wild type strain (Fig. 3Aii). Activation of platelets by whole *S. aureus* *ΔdltABCD* was indistinguishable from the parent (results not shown), thus it appears that LTA needs to be released from *S. aureus* to exert its inhibitory effect.

### **LTA inhibits [Ca<sup>2+</sup>] mobilization**

During the initial stages of platelet activation, intracellular calcium stores are mobilized to modulate downstream signalling. In order to further characterize the effect of LTA on platelets during the early stages of aggregation, an assay to determine intracellular calcium mobilisation and influx was performed. Platelets

pre-incubated with LTA showed a significantly reduced ability to mobilise calcium when challenged with CRP-XL compared to a vehicle treated control ( $P < 0.001$ ) (Fig. 3Bi). Over the course of the assay this equated to a 70% reduction in intracellular calcium levels (Fig. 3Bii). Because of its reduced inhibitory potency, calcium release stimulated by LTA from SA113  $\Delta dltABCD$  was assayed. From the mean trace of fluorescence over the course of the experiment a repeatable difference was observed (Fig. 3Bi). The peak end point fluorescence caused by LTA derived from wild type and  $\Delta dltABCD$  strains were significantly different (Fig. 3Bii).

#### **LTA inhibition of platelet activation can be blocked using anti-Paf receptor antibodies and Ginkgolide B**

In different cell types, LTA has previously been reported to bind four receptors CD14[21], CD36[22], TLR2[23] and platelet activating factor receptor (PafR)[24]. Monoclonal antibodies with blocking activity for TLR2 and CD14, along with anti-PafR and -CD36 monoclonal antibodies were each tested for their ability to block LTA inhibition (Fig. 4Ai). The anti-CD14 and -PafR antibodies were isotype matched. No significant blocking of LTA inhibition occurred with the anti-CD14, -CD36, -TLR2 or mouse IgG (negative control). However anti-PafR abolished LTA-mediated inhibition (Fig 4Ai, Aii). Furthermore, ginkgolide B, a specific PafR antagonist [25] blocked LTA-mediated platelet inhibition (Fig. 4Bi), reducing it to 0% inhibition (Fig. 4Bii). These data demonstrate a role for PafR on platelets as an LTA receptor.

Using flow cytometry, anti-PafR antibody abolished binding of FITC labelled LTA to platelets, demonstrating that PafR is the only receptor for the molecule (Fig. 4C).

To test whether anti-PafR antibody blocking was due to a non-specific interaction with LTA, rather than blocking of PafR. We were unable to deplete samples of their inhibitory effect using anti-PafR antibodies, linked to protein A coated magnetic beads (results not shown). The blocking effect of anti-PafR was not due to cross-reactivity with LTA.

#### **LTA causes raised cAMP levels to cause inhibition of platelet activation**

Our finding that LTA inhibited  $\text{Ca}^{2+}$  flux in platelets led us to hypothesise that an increase of cAMP would occur in platelets upon incubation with LTA, which could be blocked with ginkgolide B. Increased cAMP concentrations attenuate  $\text{Ca}^{2+}$  mobilisation, which is necessary for platelet activation. In platelets, raised levels of cAMP result in increased levels of phosphorylated vasodilator-stimulated phosphoprotein (VASP)[26]. Qualitative assessment of VASP phosphorylation was carried out by western blot. Samples were prepared that had been pre-treated with ginkgolide B, incubated with LTA and were compared with control samples (Fig. 5A). Levels of phosphorylated VASP, shown by the upper band which represents phosphorylation at residue Ser 157[26] were increased in both the positive control (prostacyclin treated) and LTA treated samples indicating a possible role for increased platelet cAMP concentrations in LTA mediated inhibition. To confirm that this inhibition occurred via PafR, two samples were treated with ginkgolide B and one of these with LTA also. In both

samples no increase in VASP phosphorylation was observed, demonstrating that VASP phosphorylation resulting from LTA treatment can be blocked by the PafR antagonist. Furthermore, supernatant from *S. aureus* SEJ1 and SEJ1  $\Delta gdpP$  were able to cause VASP phosphorylation. Supernatant from *S. aureus* SEJ1  $\Delta gdpP$   $\Delta ltaS$  was not able to induce phosphorylation, but the effect was restored in supernatant of the complemented strain (Fig. 5A). These data provide genetic proof of the role of LTA in VASP phosphorylation.

The cAMP concentration in platelet lysates was assayed (Fig. 5B). A mean *c.* 350% increase of cAMP concentration occurred in platelets incubated with LTA. In platelets pretreated with ginkgolide B, no increase was observed, correlating with the results from the western blot for VASP phosphorylation. No increase in VASP phosphorylation or cAMP levels was observed in samples treated solely with ginkgolide B.

#### **LTA reduces platelet thrombus formation *in vitro* and causes extended bleeding time *in vivo*.**

In order to investigate whether LTA could inhibit thrombus formation in whole blood and under arterial flow conditions, whole human blood was perfused through collagen coated biochips in the presence of tyrodes buffer and LTA from wild type *S. aureus* or from *S. aureus* SA113  $\Delta dltABCD$  (Fig. 6A). In blood pretreated with LTA, a significantly reduced thrombus size, compared to the vehicle treated control, was observed ( $P < 0.001$ ) (Fig. 6A, 6B). Additionally peak fluorescence was reduced by approximately 85% (Fig. 6C). Blood treated with LTA extracted from SA113  $\Delta dltABCD$  showed a slower rate of thrombus

formation however at the end of the ten minutes the thrombi present were no different in size than vehicle treated ( $P>0.05$ ) (Fig. 6B). The mean peak fluorescence of thrombi formed in the presence of LTA from *S. aureus*  $\Delta dltABCD$  showed no difference to that of the control but was significantly different than wild type LTA treated ( $P<0.05$ ) (Fig. 6C). Inhibition of thrombus formation in blood was consistent with the reduced platelet function observed in washed platelets (Fig. 1Ai – Eii).

To substantiate these findings, an *in vivo* mouse model of bleeding was used. Previous reports have proposed that mouse platelets may lack PafR[27], however by Western blotting, we identified a *c.* 48 kDa band in mouse platelets that co-migrates with PafR on human platelets (Fig. S3A). Similarly, Paf was a weak agonist, compared to collagen, for mouse and human platelet activation (Fig. S3B) and LTA inhibited platelet activation by collagen in mouse platelets (Fig. S3C). The effect of LTA on maintenance of haemostasis was measured by a tail bleed assay. Infusion of *S. aureus* LTA into rodents does not induce shock or affect blood pressure [28]. The mean bleeding time of vehicle treated (PBS) mice was 340 s following tail biopsy (Fig. 7). In LTA treated mice mean time to cessation of bleeding increased significantly ( $P<0.01$ ), more than doubling to 690 s.

LTA has multiple roles on many different host cell types and we cannot rule out an additional role for the endothelium in the tail bleeding experiments, but by measuring thrombus formation in the absence of endothelial cells, we have confirmed a role for LTA alteration of platelet function in thrombus formation.

Taken together these data demonstrate that LTA has a role in the inhibition of thrombosis and haemostasis.

## Discussion

The interplay between *S. aureus* and its human host is complex. Numerous host and pathogen factors are involved in the interaction and many have multiple activities. *S. aureus* LTA is a polymeric glycerol-phosphate molecule that can be fixed to the cell membrane by a lipid anchor and has a well-established role in several host-pathogen interactions. Although attached to the cell envelope, LTA is also released into the *S. aureus* supernatant, a process accelerated by some antibiotics[29]. *In vivo* during *S. aureus* infections, LTA has been detected, albeit by a different method to ours, at up to 10 µg/mL[30]. *S. aureus* modifies LTA with D-alanine, which confers multiple effects on its function. Interestingly, the presence of D-alanine on *S. aureus* teichoic acids confers resistance to platelet microbicidal protein, a product of activated platelets[31]. We have shown that d-alanylation increases the platelet-inhibitory potency of LTA. Thus the *dltABCD* operon plays a dual role in protecting *S. aureus* against the bactericidal effects of platelet activation.

Interestingly, LTA from wild type *S. aureus* was more inhibitory than that from the isogenic *dltABCD* mutant. However there was no difference in the ability of *S. aureus* cells to induce platelet activation in the absence of exogenous LTA.

Both D-alanine residues and lipid anchor have been reported to be required for stimulation of cytokine production in human whole blood and mouse monocytes[32]. This may explain our observation, as the lipid, which is usually

embedded within the bacterial membrane, would only be exposed to host cell receptors upon release of LTA. Alternatively, measurements of the *S. aureus* cell wall show it to be sufficiently thick that the commonly depicted schematic in which LTA chains extend through the cell wall and are exposed on the surface, may be incorrect[33].

Many of the ascribed functions of LTA have been determined using commercial preparations[24,34], which were subsequently shown to contain contaminants responsible for the activity[10,18,35]. Thus results obtained using such preparations may remain open to question. We confirmed that properly purified LTA does inhibit platelet activation. Moreover LTA inhibits activation of platelets by multiple physiological agonists and whole *S. aureus* cells, each of which trigger activation in different ways, suggesting that LTA blocks a common downstream effect.  $Ca^{2+}$  mobilization, a critical stage in platelet activation, was inhibited and is accompanied by increased cellular cAMP concentrations. LTA interacts with PafR, a cell surface receptor that couples to different G proteins to activate cellular responses that differ between cell types[36-40]. In platelets, the G-proteins that interact with PafR remain to be determined. However PafR does not signal through the pertussis toxin-sensitive  $G_i$  and  $G_o$  proteins in platelets[41]. It is well documented that platelet GPCRs can influence cellular cAMP concentrations, thereby inhibiting  $Ca^{2+}$  flux[42]. In other cells, PafR interacts with multiple G proteins, resulting in activation of distinct signalling pathways. Indeed, differential PafR signalling in response to agonists and inverse agonists has been reported[43]. Leukocyte responses to Paf utilise pertussis toxin-insensitive and -sensitive G protein(s)[44,45]. In CHO cells, Paf activation of p38

MAPK occurs through G<sub>q</sub> protein, but Paf activation of extracellular signal-regulated kinases 1 and 2 occurs via signalling through G<sub>o</sub> protein[46]. PafR signalling in HUVECs, including cAMP production by stimulation of adenylate cyclase, occurs via the G<sub>q</sub> protein[47]. The exact nature of the signalling events that lead to the increased platelet cAMP levels will be the topic of future pharmacological studies.

The ability of *S. aureus* to induce platelet activation is well documented[3] and is presumed to be important in the development of bacterial endocarditis. *S. aureus* therefore possesses the ability both to positively and to negatively influence thrombus formation. It seems questionable that platelet activation is advantageous for any bacteria. In doing so, a pathogen becomes enmeshed in a thrombus that can lead to the death of the host, leaving the bacterium unable to continue the infectious cycle. Indeed, the ability to inhibit platelet activation presumably confers an advantage to pathogens during infection. Rich sources of bioactive molecules, some of which are bactericidal, platelets have roles in modulating other cellular functions, including those of the innate and acquired immune systems[48] and as such serve to alert the host to the presence of an infection. Activated platelets can engulf *S. aureus*, although whether this occurs *in vivo* or has any role in infection remains to be determined[49]. Furthermore, direct interaction with activated platelets induces hyperactivation of neutrophils, enhancing their already potent antibacterial activity[50]\_ENREF\_65.

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## Figure legends

**Figure 1. LTA from *S. aureus* inhibits platelet aggregation.** Washed human platelets ( $4 \times 10^8$  cells/mL) or PRP were pre-incubated with LTA or tyrodes buffer and stimulated with various platelet agonists. Aggregation was measured as change in light transmission. (Ai-Di) Representative aggregation traces of platelets incubated with LTA and stimulated with various platelet agonists. All aggregation traces commence upon addition of agonist. (Aii-Dii) Data are plotted as percentage inhibition of aggregation or percentage aggregation (vehicle treated representing 100% aggregation) and represent mean values + SEM. (A) Platelets were pre-incubated with LTA at a range of concentrations followed by stimulation with CRP-XL ( $0.5\mu\text{g/mL}$ ). Aggregation was measured for 90s. (B) Platelets were pre-incubated with LTA at a range of concentrations followed by stimulation with Paf ( $37.5\mu\text{g/mL}$ ). Aggregation was measured for 90s. (C) Platelets were pre-incubated with LTA at a range of concentrations followed by stimulation with thrombin ( $0.05$  units/mL). Aggregation was measured for 90s. (D) Platelets were pre-incubated with LTA ( $4\mu\text{g/mL}$ ) for 8, 10, 12, 14 and 16min followed by stimulation with CRP-XL ( $0.5\mu\text{g/mL}$ ). Aggregation was measured for 90 s. (E) PRP was pre-incubated with various concentrations of LTA for 15min followed by stimulation with whole *S. aureus* SA113 cells ( $2 \times 10^8$  cells/mL). Aggregation was measured for up to 20min. Data are plotted as increase in lag time and represent mean values + SEM. \* $P < 0.05$

**Figure 2. The inhibition is due to the presence of *S. aureus* LTA.** (A) Washed human platelets ( $4 \times 10^8$  cells/mL) were pre-incubated with anti-LTA

antibody (1 µg/mL) before incubation for 15min with LTA (2 µg/mL) followed by stimulation with CRP-XL (0.5 µg/mL). Aggregation was measured as change in light transmission for 90s. N.B. in Ai, lines representing platelets treated with 2 µg/mL + anti-LTA antibodies and untreated platelets, overlap extensively. (B) Washed platelets were incubated for 15min with LTA extracted from *S. aureus* SA113 (4 µg/mL), *B. subtilis* or *S. pneumoniae* D34 (12.5 µg/mL) followed by stimulation with CRP-XL (0.5 µg/mL). (C) Washed platelets were incubated for 15min with LTA extracted from *B. subtilis* at a range of concentrations followed by stimulation with CRP-XL (0.5 µg/mL). (D) Washed platelets were incubated for 15min with supernatant from *S. aureus* SEJ1, SEJ1  $\Delta gdpP$ , SEJ1  $\Delta ltaS \Delta gdpP$  or SEJ1  $\Delta ltaS \Delta gdpP$  pCN34-*ltaS* (10 µg/mL), followed by stimulation with CRP-XL (0.5 µg/mL). (Ai, Bi, Di) Representative aggregation traces of washed platelets. Aggregation was measured for 90s. (Aii, Bii, C, Dii) Data are plotted as percentage inhibition of aggregation represent mean values + SEM. \*P<0.05, \*\*P<0.001

**Figure 3. LTA from *S. aureus*  $\Delta dltABCD$  has a reduced ability to inhibit**

**platelet aggregation.** (A) Washed human platelets ( $4 \times 10^8$  cells/mL) were incubated for 15min with LTA extracted from *S. aureus* strains SA113 or SA113  $\Delta dltABCD$  (4 µg/mL) followed by stimulation with CRP-XL (0.5 µg/mL). (B) Platelets in PRP were pre-loaded with Fluo-4NW dye. Platelets were then pre-incubated with LTA extracted from *S. aureus* strains SA113 or SA113  $\Delta dltABCD$  (4 µg/mL) or Tyrodes buffer for 15min followed by stimulation by CRP-XL (0.5 µg/mL). Intracellular mobilization of calcium was measured by spectrofluorimetry for 120s. (Ai) Representative aggregation traces of washed

platelets. Aggregation was measured for 90s. (Ai) Representative aggregation trace. (Aii) Data are plotted as percentage maximum fluorescence (vehicle treated representing 100% aggregation) and represent mean values + SEM. (Bi) Representative calcium flux trace. (Bii) Data are plotted as percentage endpoint fluorescence (vehicle treated representing 100% aggregation) and represent mean values + SEM. \* $<0.01$ , \*\* $P<0.005$

**Figure 4. LTA acts through PafR to inhibit platelets.** (A) Washed platelets were incubated with anti-PafR (4 $\mu\text{g}/\text{mL}$ ), anti-TLR2 (4 $\mu\text{g}/\text{mL}$ ), anti-CD14 (4 $\mu\text{g}/\text{mL}$ ), Mouse IgG (4 $\mu\text{g}/\text{mL}$ ) or Tyrodes buffer for 30min before addition of LTA (4 $\mu\text{g}/\text{mL}$ ) for 15min. (B) Washed platelets were incubated with Ginkgolide B (2mM) or Tyrodes buffer for 30min before the addition of LTA (4 $\mu\text{g}/\text{mL}$ ) for 15min. Platelets were then stimulated with CRP-XL (0.5 $\mu\text{g}/\text{mL}$ ). N.B. in Bi, lines representing platelets treated with 4 $\mu\text{g}/\text{mL}$  LTA + 2 $\mu\text{M}$  Ginkgolide B and 0 $\mu\text{g mL}^{-1}$  LTA + 2 $\mu\text{M}$  Ginkgolide B, overlap extensively. (A-B) Platelet aggregation was measured as change in light transmission and recorded for 90s. Data are plotted as percentage inhibition of aggregation (normalised so that LTA treatment represents 100% inhibition) and represent mean values +SEM. \* $P<0.0001$ , \*\* $P<0.01$ . (C) PRP (4 x 10<sup>8</sup> cells/mL) was incubated for 30min with anti-PafR (50 $\mu\text{g}/\text{mL}$ ), IgG2<sub>a</sub> (50 $\mu\text{g}/\text{mL}$ ) or Tyrode's buffer before the addition of FITC-LTA at several concentrations for 15 mins. Samples were run through a BD Accuri<sup>TM</sup> C6 flow cytometer and median fluorescence was recorded. Data are plotted as percentage median increase in fluorescence when compared to a Tyrode's buffer only control and represent mean values +SEM.

**Figure 5. Incubation of platelets with LTA increases cAMP concentrations.**

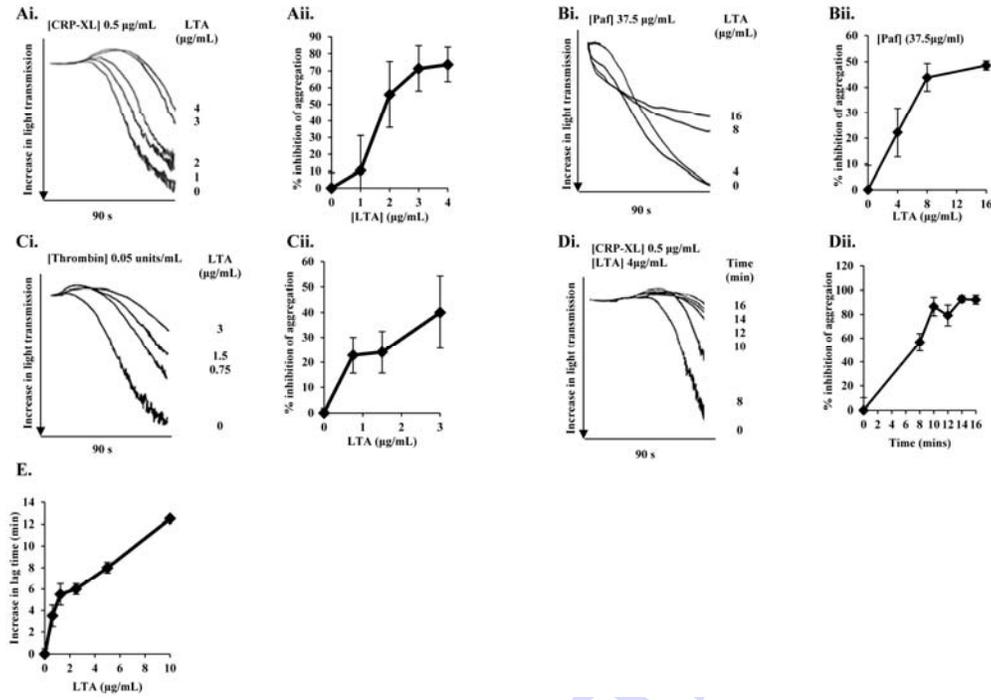
(A) Platelets ( $8 \times 10^8$  cells/mL) were pre-treated with either Ginkgolide B (2mM) or tyrodes buffer for 30min. Platelets were then treated with LTA from *S. aureus* SA113 (4 $\mu$ g/mL), PGI<sub>2</sub> (0.25 $\mu$ g/mL), supernatant from *S. aureus* SEJ1, SEJ1  $\Delta gdpP$ , SEJ1  $\Delta ltaS \Delta gdpP$  or SEJ1  $\Delta ltaS \Delta gdpP$  pCN34-*ltaS* (10 $\mu$ g/mL) or Tyrodes buffer. Lysates were immuno-blotted with an anti-VASP antibody. (B) Platelets ( $8 \times 10^8$  cells/mL) were pre-treated with either Ginkgolide B (2mM) or tyrodes buffer for 30min. Platelets were then treated with LTA (4 $\mu$ g/mL), PGI<sub>2</sub> (0.25 $\mu$ g/mL) or tyrodes buffer. Samples were then assayed for cAMP concentration determined by ELISA. Data represent mean values +SEM. \*P<0.05

**Figure 6. LTA inhibits thrombus formation *in vitro* and haemostasis *in vivo*.**

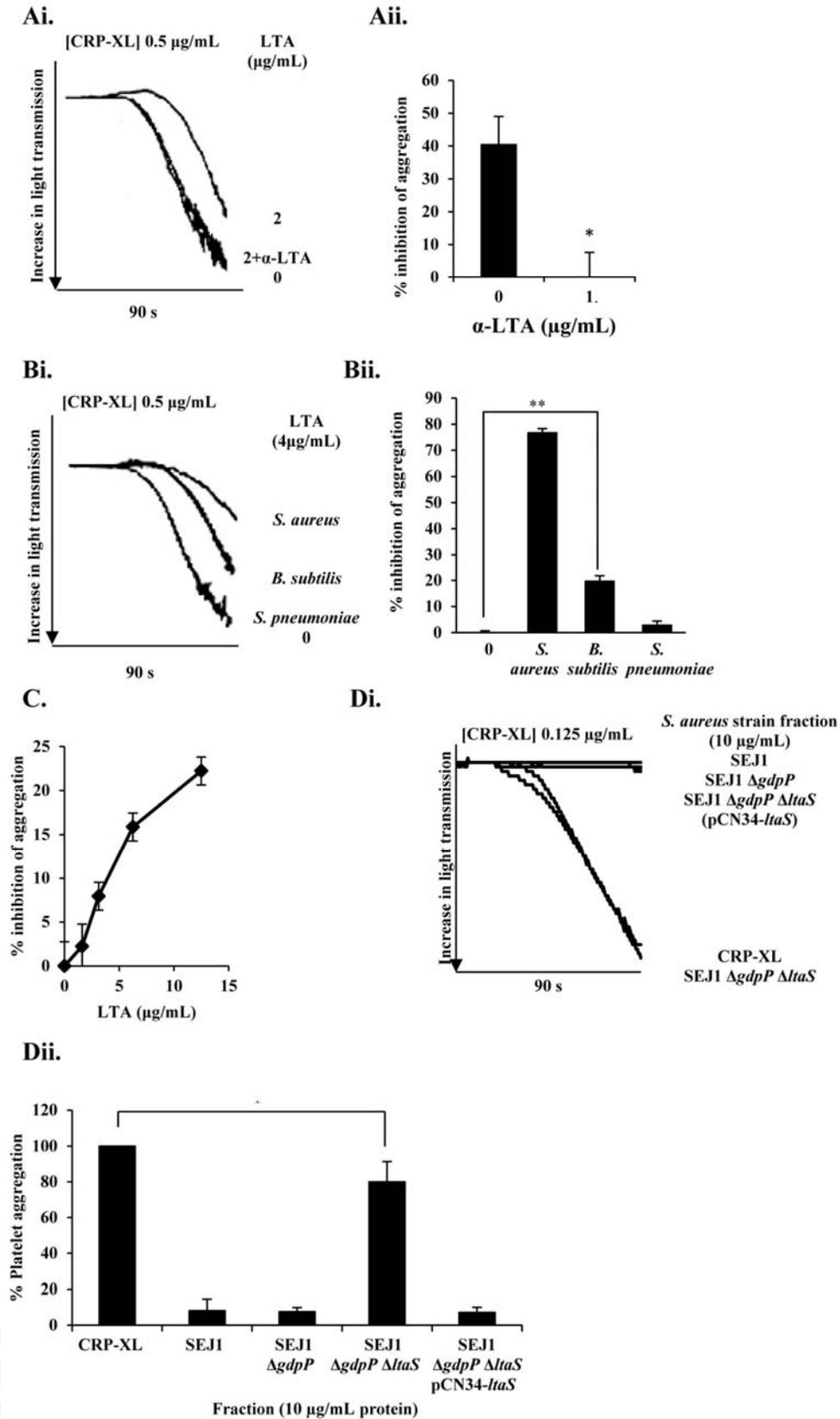
(A) Platelets within whole human blood were labelled with a lipophilic dye DIOC6. Whole blood was then treated with (Ai) tyrodes buffer, (Aii) LTA extracted from *S. aureus* strains SA113 (10 $\mu$ g/mL) or (Aiii) LTA extracted from *S. aureus* strain SA113  $\Delta dltABCD$  (10 $\mu$ g/mL) for 15min. Whole blood was then perfused through collagen coated (400 $\mu$ g/mL) Vena8™Biochip at a flow rate of 20 dynes cm<sup>-2</sup>. Formation of thrombi was recorded using a Z stack capture every 30s for 10min using a Nikon eclipse (TE2000-U) microscope. Thrombus fluorescence intensity was calculated using Slidebook™ 5 software. (B) Data represents mean of thrombi volume over the experiment duration. (C) Data represent mean +SEM of peak fluorescence intensity. \*P<0.05. \*\*P<0.01

**Figure 7. LTA affects haemostasis *in vivo*.** LTA (10µg/mL) (n=11) or PBS (n=11) was administered intravenously to mice and time to cessation of bleeding following a tail biopsy was measured. Data represent individual mice and horizontal lines refer to mean values of seconds until cessation of bleeding. \*P<0.01

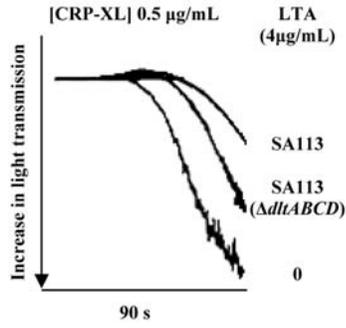
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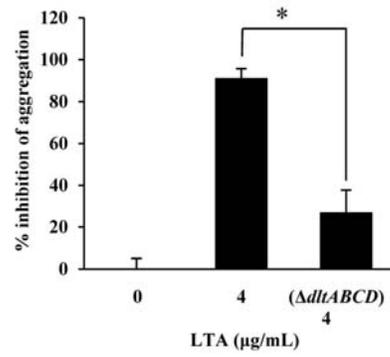
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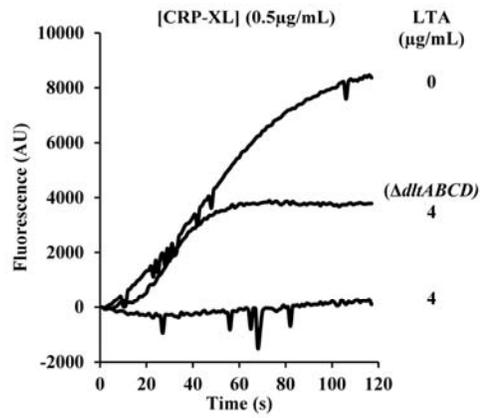
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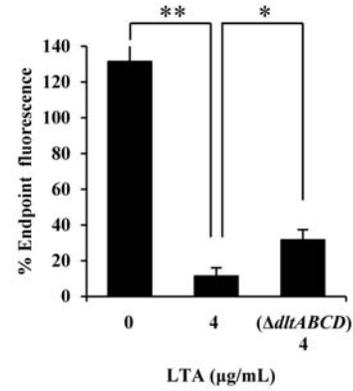
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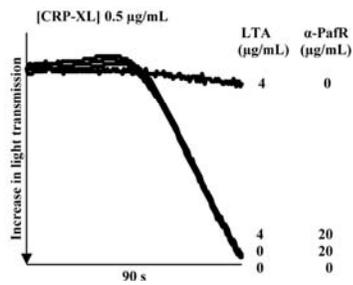


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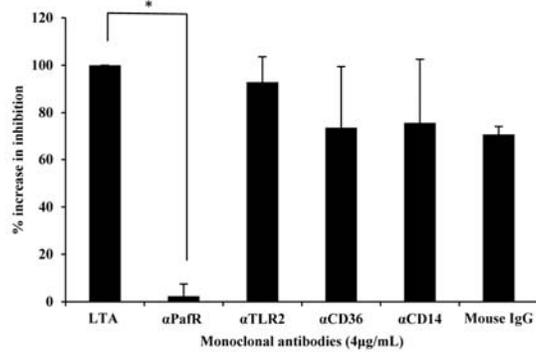


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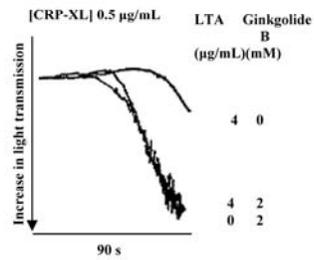
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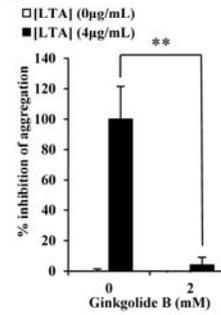
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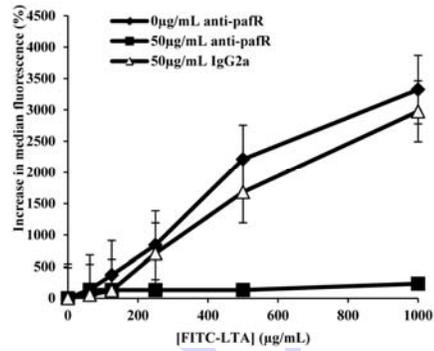
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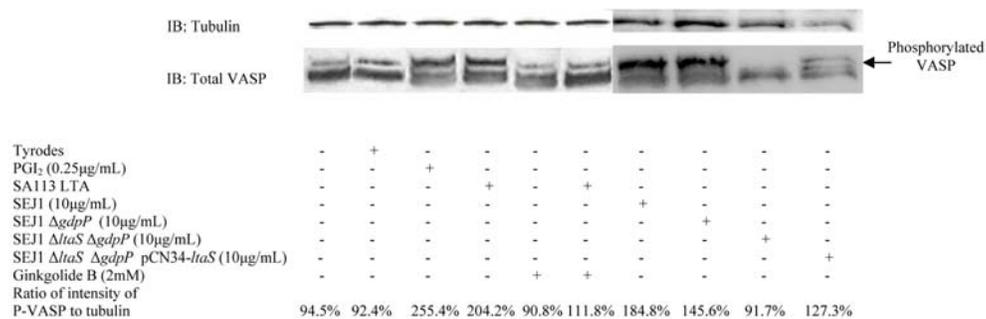
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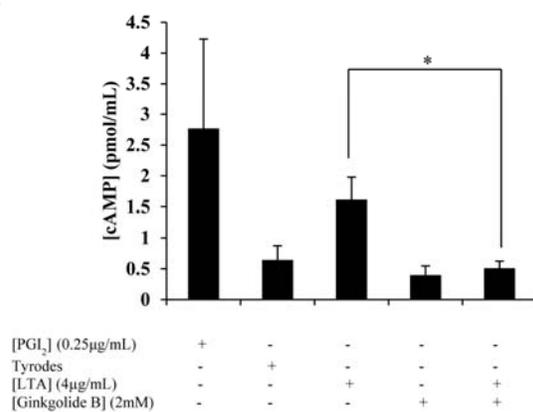
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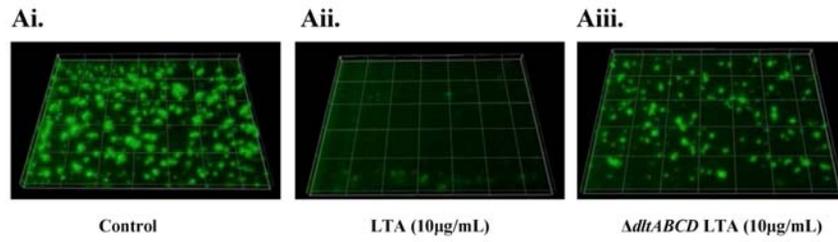
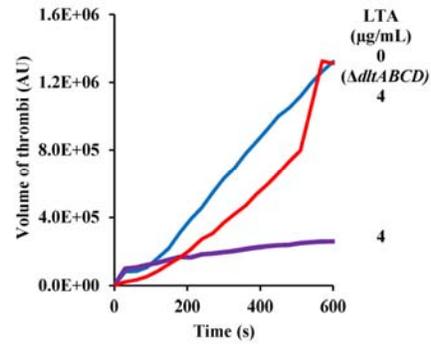
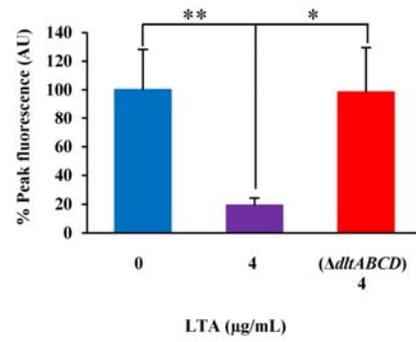
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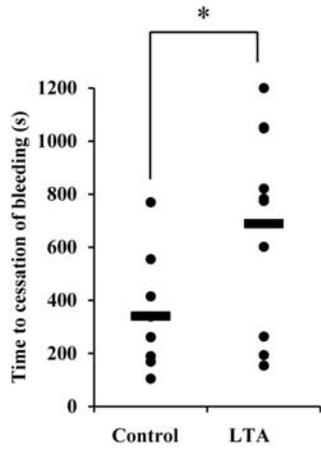
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