Modulation of platelet activity by *S. aureus* lipoproteins.

PhD Thesis

University of Reading

Pratik P. Vikhe

School of Biological Sciences

January 2016

Acknowledgements

I would like to thank my supervisor, Dr. Simon Clarke for his invaluable help, advice and patience, in producing this work. I would also like to thank my co-supervisor Prof. Jon Gibbins for his guidance and innovative experimental ideas, throughout this work.

I would like to thank Amie Waller for her vital technical and conceptual help, as well as for keeping my morale up during entire project. I would also like to thank Amanda Unsworth, Parvathy Sasikumar, Lisa Holbrook, Marilena Crescente, Neline Kriek, Tanya Sage, Alex Stainer, Alex Bye, Francoise Mazet, Shakti Vaipuri, Chris Jones and Michael Schenk for the technical advice and help given with regards to many of the techniques used within this work. I would also like to thank Gagan Flora and Khalid Sahli for their help, whenever I needed it. For help in Microbiology, I would like to thank Kevan Hansen, Silvia Loureiro and all members of CSS – Knight Building.

Finally I would like to thank Felix Scholarship for funding my PhD studies and my parents for their constant support and encouragement.

Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Pratik P. Vikhe
Date: 28 January 2016

Abstract

Staphylococcus aureus is an opportunistic pathogenic bacterium known for its ability to interact with platelets and modulate their function. *S. aureus* lipoproteins are one of the major groups of bacterial surface molecules and are released into the extracellular milieu where they are recognized by host immune cells.

The aim of this study was to determine the role of *S. aureus* lipoproteins in *S. aureus*-platelet interactions. Platelet aggregation and binding assays using *S. aureus* wild type and *lgt* strains showed that, *S. aureus* lipoproteins contribute towards binding of the pathogen to platelets. Lipoproteins present in extracellular milieu also bind platelets. Platelet spreading, thrombus formation, agonist induced platelet aggregation and αIIbβ3 activation were inhibited by cell-free lipoproteins.

CD36 was identified as the major platelet surface molecule interacting with *S. aureus* lipoproteins. Antibody neutralization demonstrated that functional inhibition of platelet activation caused by lipoproteins was via CD36. Pre-incubation of platelets with *S. aureus* lipoproteins significantly increased the association of CD36 with integrin β3. Thus CD36-β3 association is proposed to be important in inhibiting activation of αIIbβ3 and consequent *S. aureus* lipoprotein mediated inhibition of platelet activation.

Table of Contents

List of Figures

List of Tables

Abbreviations

International units of measurement and biology were used throughout. Other abbreviations used are:

°C – Degrees Celcius

ACD – Acid citrate dextrose

ANOVA – Analysis of variance

AU – arbitrary units

BHI – Brain heart infusion

BSA – Bovine serum albumin

cAMP – Cyclic adenosine monophosphate

CRP-XL – Cross linked collagen related peptide

dH_2O – De-ionised water

ECL – Enhanced chemiluminescence

EDTA – Ethylenediaminetetraacetic acid

EF hand motif – Calcium binding motif made of two helices – E and F

FITC – Fluorescein isothiocyanate

g – Gram

GPx - Glycoprotein

hr – Hour

HRP – Horse radish peroxidase

kDa – Kilodalton

L – Litre

LTA – Lipoteichoic acid

M – Molar

MSCRAMM – Microbial surface component recognising adhesive matrix molecules

mins – Minutes

OD_x – Optical density at x nanometers wavelength

PAGE – Polyacrylamide gel electrophoresis

PBS – Phosphate buffered saline

PBST – Phosphate buffered saline with tween

PGI_2 – Prostacyclin

PMSF – Phenylmethylsulfonyl fluoride

PRP – Platelet rich plasma

PVDF - Polyvinylidene fluoride

ROI – Reactive oxygen intermediates

sec – Seconds

SDS – Sodium dodecyl sulphate

SEM – Standard error of the mean

TBS – Tris buffered saline

TBST – Tris buffered saline with tween

TEMED – N, N, N' N'-Tetramethyl-ethylenediamine

TLRx – Toll-like receptor

VASP - Vasodilator-stimulated phosphoprotein

P-VASP – Phosphorylated vasodilator-stimulated phosphoprotein

vWF – Von willebrand factor

Chapter 1
Introduction

The *Staphylococci* are group of facultative anaerobic Gram-positive cocci, which grow in grape like clusters (Bhatia & Zahoor 2007). They are 0.5 to 1.5µm in diameter with a cell wall composed of peptidoglycan and teichoic acid (Coley *et al*. 1976). Most of the bacteria in this genus are part of the normal human microbiota and are inhabitants of the upper respiratory tract (Pettigrew *et al*. 2008), skin (Chiller *et al*. 2001), gut (Sannasiddappa *et al*. 2011) and vagina (Weinstein 1938). Some are opportunistic pathogens and belong to the group of invasive Gram-positive pyogenic cocci (Finlay & Falkow 1997). Based on their ability to produce coagulase that can convert fibrinogen to fibrin (Tager 1974), *Staphylococci* are classified as coagulase positive or negative (Baird-Parker 1963). Some *Staphylococci* produce capsule and slime which gives this bacterium the ability to evade immune response by inhibiting neutrophil chemotaxis and phagocytosis (Barrio *et al*. 2000; Johnson *et al*. 1986). Slime also helps in providing resistance against antimicrobial agents (Souli *et al.* 1998).

1.1. *Staphylococcus aureus*

Staphylococcus aureus is a major pathogenic bacterium of humans and animals. Most of *S. aureus* ("aureus" is latin for golden) strains give golden colonies on the rich medium due to production of yellow pigment, staphyloxanthin (Pelz *et al*. 2005). Staphyloxanthin gives *S. aureus* the ability to grow under condition of osmotic pressure and low moisture (Clauditz *et al*. 2006). These abilities aid *S. aureus* in colonisation of nasal secretions and skin. *S. aureus* uses different strategies to adhere, invade and survive in the host.

1.1.1. *S. aureus* adhesion strategies

S. aureus expresses different surface proteins like fibronectin binding proteins (FnBP) (Roche *et al*. 2004), clumping factors A and B (L. O'Brien *et al*. 2002), Serine-Aspartate Repeat Proteins (Sdr) (Sabat *et al*. 2006), *S. aureus* Protein A (SpA) (Kobayashi & DeLeo 2013), Iron regulated surface determinant protein A (IsdA) (Clarke *et al*. 2009), which aid in its adhesion to host surfaces (Figure 1.1). FnBPs are one of the important microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (O'Neill *et al*. 2008) and can bind to fibronectin (O'Neill *et al*. 2008), fibrinogen and elastin (Keane *et al*. 2007). FnBPs also interact with integrin α5β1 on host cells via a fibronectin bridge, which allows *S. aureus* to bind and invade endothelial cells (Schröder *et al*. 2006), epithelial cells (Medina-Estrada *et al*. 2015), fibroblasts and keratinocytes (Kintarak *et al*. 2004). *S. aureus* strains deficient in FnBPs lack invasiveness and ability to cause sepsis (Edwards *et al*. 2010). FnBPs are associated with biofilm formation capabilities of *S. aureus* MRSA strains (O'Neill *et al*. 2008).

S. aureus expresses clumping factors (Clf) which can bind to fibrinogen (Ní Eidhin *et al*. 1998). Two clumping factors are produced by *S. aureus* – ClfA and B (Foster *et al.* 1998), which have different binding abilities. ClfA binds to the extreme C terminus of the γ-chain (McDevitt *et al*. 1997), whereas ClfB binds to the α-chain of fibrinogen (Ní Eidhin *et al*. 1998). ClfA binding to fibrinogen in blood plasma is considered a significant virulence factor in septic arthritis and infective endocarditis (Ganesh *et al*. 2008). ClfA also binds to complement factor and protects *S. aureus* from phagocytosis (Hair *et al*. 2008). ClfB is implicated in adhesion of nasal epithelium by binding to epidermal cytokeratin's (O'Brien *et al*.

2002) and squamous epithelial cell envelope protein loricrin (Mulcahy *et al*. 2012). ClfB binds to dermokine, thus showing binding ability to multiple ligands (Xiang *et al*. 2012).

Another important set of MCRAMMs are the Serine-Aspartate Repeat Proteins (Sdr) (Foster *et al.* 1998). Three Sdr proteins are expressed by *S. aureus* - Sdr C, D and E (Sabat *et al*. 2006). SdrC binds to cultured mammalian cells expressing β-neurexin (Barbu *et al*. 2010). SdrC is also involved in biofilm formation (Barbu *et al*. 2014). Under low iron conditions (found *in–vivo*), *S. aureus* expresses IsdA proteins (Pishchany *et al*. 2009). IsdA has broad spectrum binding ability (Clarke *et al*. 2004). Along with fibrinogen and fibronectin, it can bind involucrin, loricrin, and cytokeratin K10 (Clarke *et al*. 2009), proteins that are present in the cornified envelope of human desquamated epithelial cells. Along with binding to cellular and extracellular matrix components, IsdA binds heme (Pishchany *et al*. 2009) and is also known to regulate innate immune response against *S. aureus* (Foster *et al*. 2014).

S. aureus expresses teichoic acid, a highly charged molecule on its surface (Gross *et al*. 2001). Teichoic acids play important roles in binding of *S. aureus* to nasal epithelial cells (Aly *et al*. 1980). Wall teichoic acid on the surface of *S. aureus* is significant in binding to epithelial cells (Baur *et al*. 2014) and wall teichoic acid deficient *S. aureus* strains show an inability to cause endocarditis in rabbits (Weidenmaier *et al*. 2005). The charge distribution caused by teichoic acid is speculated to be important in binding of *S. aureus* to artificial surface (e.g. surgical or medical devices) (Gross *et al*. 2001). Wall teichoic acids on the *S. aureus* surface also confer immune resistance by blocking binding of

antimicrobial fatty acids (Kohler *et al*. 2009) and antibodies to the surface epitopes on *S. aureus* (Gautam *et al*. 2015).

1.1.2. *S. aureus* invasion strategies

S. aureus breaches host protective barriers by secreting different toxins. These include toxic shock syndrome toxin 1 (TSST-1), exfoliative toxins (ETs) and haemolysins (Johnson *et al*. 1991). Toxic shock syndrome toxin 1 (TSST-1) is the major cause of Toxic shock syndrome, which is characterized by fever, rash, hypotension, multiple-organ-system dysfunction and desquamation (Parsonnet *et al.* 2008). TSST-1 is a pyrogenic superantigen (Wahlsten *et al.* 1998). It stimulates T- cell and monocyte mediated massive cytokine (TNF and IL-1) release resulting in hyper immune response and cellular damage (Miethke *et al.* 1993). Exfoliative toxins are the major factor in pathogenesis of scalded skin syndrome and one of the significant factors in aiding entry of bacteria into blood (Bukowski *et al*. 2010). These toxins are proteases and act on desmoglein-1, a protein that plays an important role in maintaining cell-to-cell adhesion in the superficial epidermis (Ladhani 2003).

Other toxins are haemolysins among which, the most extensively studied is α-haemolysin (α-toxin), which forms pores in host cells (skin cells and RBC's) (Wiseman 1975). *S. aureus* strains deficient in α-toxin are attenuated for invasiveness (Patel *et al*. 1987). These toxins can form small pores which result in cellular damage due to osmotic imbalance (Krasilnikov *et al.* 1988). Along with that, the pores caused by these toxins also result in secondary effects like programmed cell death (Bantel *et al.* 2001), signalling, secretory and cellular

contractile dysfunction due to calcium ion mobilization and release of cytokines (Brauweiler *et al*. 2014; Bhakdi *et al.* 1991).

S. aureus also produces other proteins that act as superantigens and cause cellular damage using the host immune system*. S. aureus* proteins like protein A (Kobayashi & DeLeo 2013) and extracellular fibrinogen binding proteins (Efb) (Georgoutsou-Spyridonos *et al*. 2015) modulate immune function. SpA, released into the supernatant can alter the function of B-cells and T-cells (Becker *et al*. 2014).

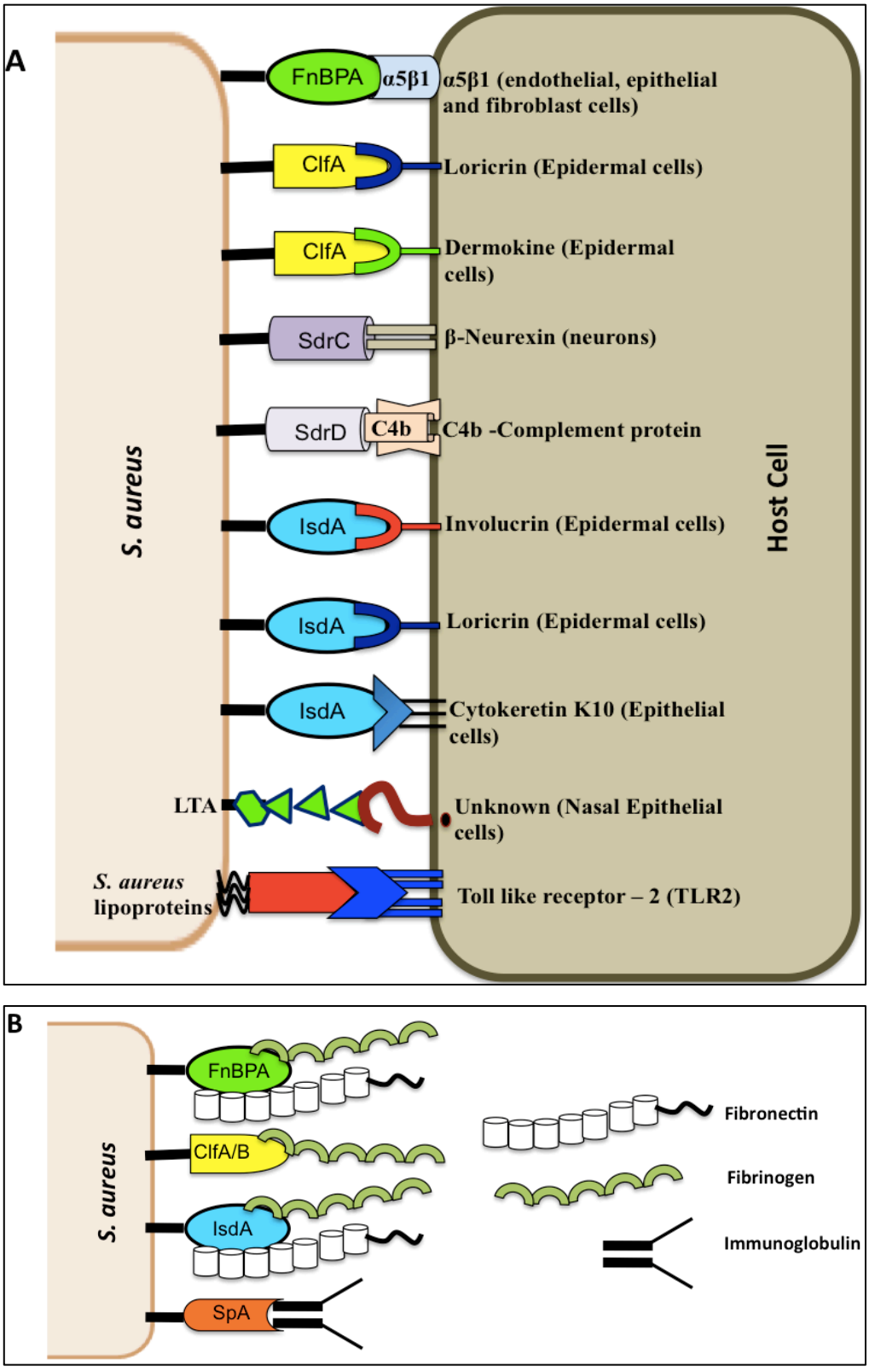

Figure 1.1: Interaction between* S. aureus *surface proteins with host proteins. ***A.*** S. aureus *surface proteins adhering with host cell proteins playing significant part in* S. aureus *adhesion and evasion during infection.* ***B.*** S. aureus *surface proteins interacting with soluble host proteins, playing significant part in forming bridging molecules.*

1.1.3. *S. aureus* evasion strategies

Neutrophil phagocytosis is the most efficient way to clear an *S. aureus* infection (Rigby & DeLeo 2012). The pathogen has developed many anti – phagocytosis strategies (Figure 1.2). It can produce a capsule rich in polysaccharide that can block phagocytosis and help *S. aureus* to survive in blood (O'Riordan & Lee 2004). *S. aureus* produces proteins that bind to antibody and complement inhibiting their function. *S. aureus* protein A binds the Fc portion of antibody (Atkins *et al*. 2008) and extracellular fibrinogen binding proteins (Efb) can bind to complement proteins C3b (Lee *et al*. 2004), both of these mechanisms aid *S. aureus* evasion of phagocytosis. SdrD protein also contributes to immune evasion by binding complement factors (Foster *et al*. 2014).

S. aureus produces staphylokinase which can cleave human plasminogen to form plasmin which then acts as a bacterial surface-bound serein protease, which can cleave IgG and C3b at the bacterial surface, thus inhibiting phagocytosis (Rooijakkers *et al*. 2005). *S. aureus* has also developed strategies to survive in phagocytic cell (Gresham *et al*. 2000). It can neutralize Reactive Oxygen Species (ROI's) by producing catalase and superoxide dismutase (Mandell 1975) and can produce toxins, like phenol-soluble modulins and leukocidin AB, that lyse the neutrophil after phagocytosis (DuMont *et al*. 2013; Surewaard *et al*. 2013). This ability of *S. aureus* helps it to survive and spread in different sites of the body using immune cells as a vehicle (Gresham *et al*. 2000). One of the efficient microbicidal activities of neutrophils is production of Neutrophil Extracellular Traps (NETs). The NETs are made of neutrophil DNA, and granular anti-microbial proteins, in which microbes are trapped and killed (Zawrotniak *et al.* 2013). *S. aureus* produces nuclease and adenosine synthase

which convert NET's to deoxyadenosine and neutralize their effect (Thammavongsa *et al*. 2013).

The ability of *S. aureus* to produce a biofilm also aids its survival and dissemination (Archer *et al*. 2011). Biofilm has an altered phenotype with regard to growth, gene expression and protein production (Donlan *et al.* 2002). On exposure to biofilm promoting conditions, like an anaerobic environment, osmotic imbalance, change in temperature and exposure to glucose, ethanol or antibiotics, *S. aureus* enhances expression of genes that promotes production of molecules essential for biofilm formation (Archer *et al.* 2011). For example, production of polysaccharide intercellular antigen (PIA), a major component in *S. aureus* biofilm, is enhanced in anaerobic conditions (Cramton *et al.* 2001). *S. aureus* biofilms are associated with diseases like osteomyelitis, chronic wound infections, chronic rhinosinusitis, endocarditis, ocular infections and poly microbial biofilm infections (Figure 1.3) (Archer *et al.* 2011). The efficient biofilm formation ability also aids in survival of *S. aureus* on medical devices like implants, prosthetic joints and heart valves, making it a leading cause of nosocomial infections (Valaperta *et al.* 2010).

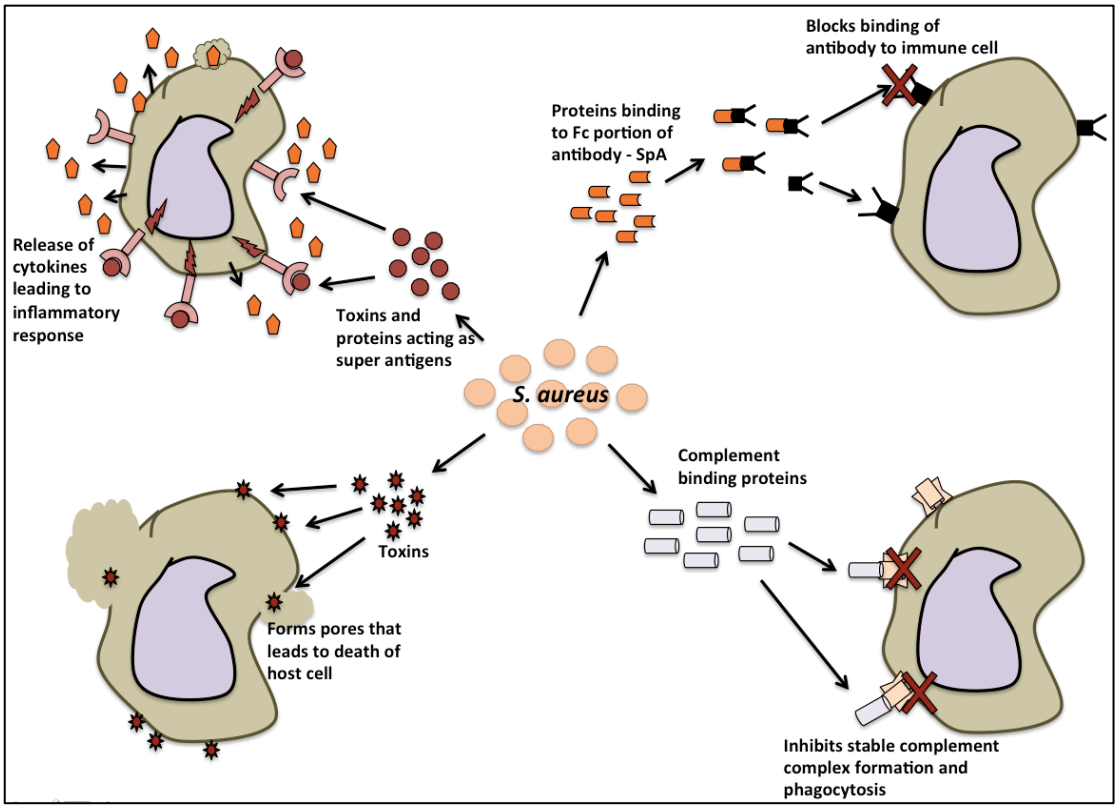

Figure 1.2:* S. aureus *evasion strategies. S. aureus *produce proteins that can bind the Fc portion of antibody and complement components thus inhibit antibody and complement mediated phagocytosis. Toxins produced by* S. aureus *can form pores and also act as super antigens causing host cell lysis and hyper immune response leading to damage to host cell.*

1.2. *S. aureus* infections

S. aureus is an opportunistic pathogen that uses multiple strategies to adhere, invade and survive in the host and it has the ability to cause infection at numerous sites in the human body (Figure 1.3). *S. aureus* is a part of the normal human skin flora, but opportunistically causes mild skin infections such as pimples, boils, impetigo, folliculitis, to severe skin infection like furuncle and necrotizing fasciitis (Lina *et al*. 1999). *S. aureus* skin infections, even though begin as minor boils or abscesses, if left untreated can progress to severe systemic diseases (McCaig *et al*. 2006).

The ability of *S. aureus* to produce enterotoxins makes it one of the leading cause of food poisoning (Bhatia *et al.* 2007), which manifests itself as nausea, violent vomiting, abdominal cramping and diarrhea (Argudín *et al.* 2010). The enterotoxins from *S. aureus* act as neurotoxins, stimulating vomiting and other symptoms (Yves *et al.* 2003; Hu *et al.* 2007).

S. aureus is also the major cause of nosocomial infections (Valaperta *et al.* 2010), it can settle on medical devices and use them as a vehicle for an entry into the host. For example, entry of *S. aureus* into bone through prosthetic joints has made it a leading cause of infection induced osteomyelitis (Olson *et al.* 2013) and *S. aureus* is speculated to be a leading cause of ventilator associated pneumonia (Wardenburg *et al*. 2007). *S. aureus* can also gain entry into the bloodstream through intravenous infusion, transducer respiratory devices, prostheses, catheters, endoscopes or via hemodialysis, all of which can lead to bacteremia (Locksley 1982). *S. aureus* bacteremia allows the pathogen to spread to different organs. Entry of the bacterium into lymph node leads to widespread seeding

causing septicemia. Also in uncommon cases *S. aureus* crosses the blood brain barrier and enters into the meninges causing meningitis (Gordon *et al.* 1985).

1.2.1. Bacterial endocarditis:

S. aureus bacteremia is often associated with the development of infective endocarditis (IE) which is characterized by the formation of vegetative growth on surface of the heart valve (L. O'Brien *et al*. 2002; Naber 2009). *S. aureus* is the leading cause of bacterial endocarditis (Naber 2009). Different mechanisms are proposed to be involved in the *S. aureus* induced endocarditis (Figure 1.4). Using numerous adhesive strategies *S. aureus* can bind to intact or damaged heart valve surface (Werden *et al*. 2014). Damage to endothelium activates the normal hemostasis system that works on to repair the damage by forming a thrombus. The thrombus consists of plasma proteins (like fibrinogen) and cells, most predominantly platelets (Werden *et al*. 2014). The platelets present on the developing thrombus are proposed to provide binding foci to the circulating *S. aureus*, thus in turn provide seeding ground for bacteria (Sullam *et al.* 1996). *S. aureus* are found to be present in the occluded thrombi associated with the infective endocarditis (Pawar *et al.* 2004).

Bacterial endocarditis is characterized by inflamed endothelium and associated lesions on the heart valve (Werden *et al*. 2014). Ability of *S. aureus* to adhere and invade host endothelium is proposed to play significant part in inflammatory lesion associated endocarditis. Super antigenicity property of *S. aureus* surface and secreted proteins leads to inflammatory response causing damage to the intact host endothelium and formation of lesions (Becker *et al.* 2014). These inflammatory lesions on heart valve provide niche for the

settlement of *S. aureus*, where they interact with circulating platelets causing their activation and aggregation, forming an infected thrombus on the valve surface (L. O'Brien *et al*. 2002). Modulation of platelet function by *S. aureus* is speculated to play significant role in pathogenesis of infective endocarditis (Ganesh *et al*. 2008). Adhesion and activation of platelets surrounding *S. aureus* aids in evasion of bacteria not only from host immune cells but also from antimicrobials (Werden *et al*. 2014).

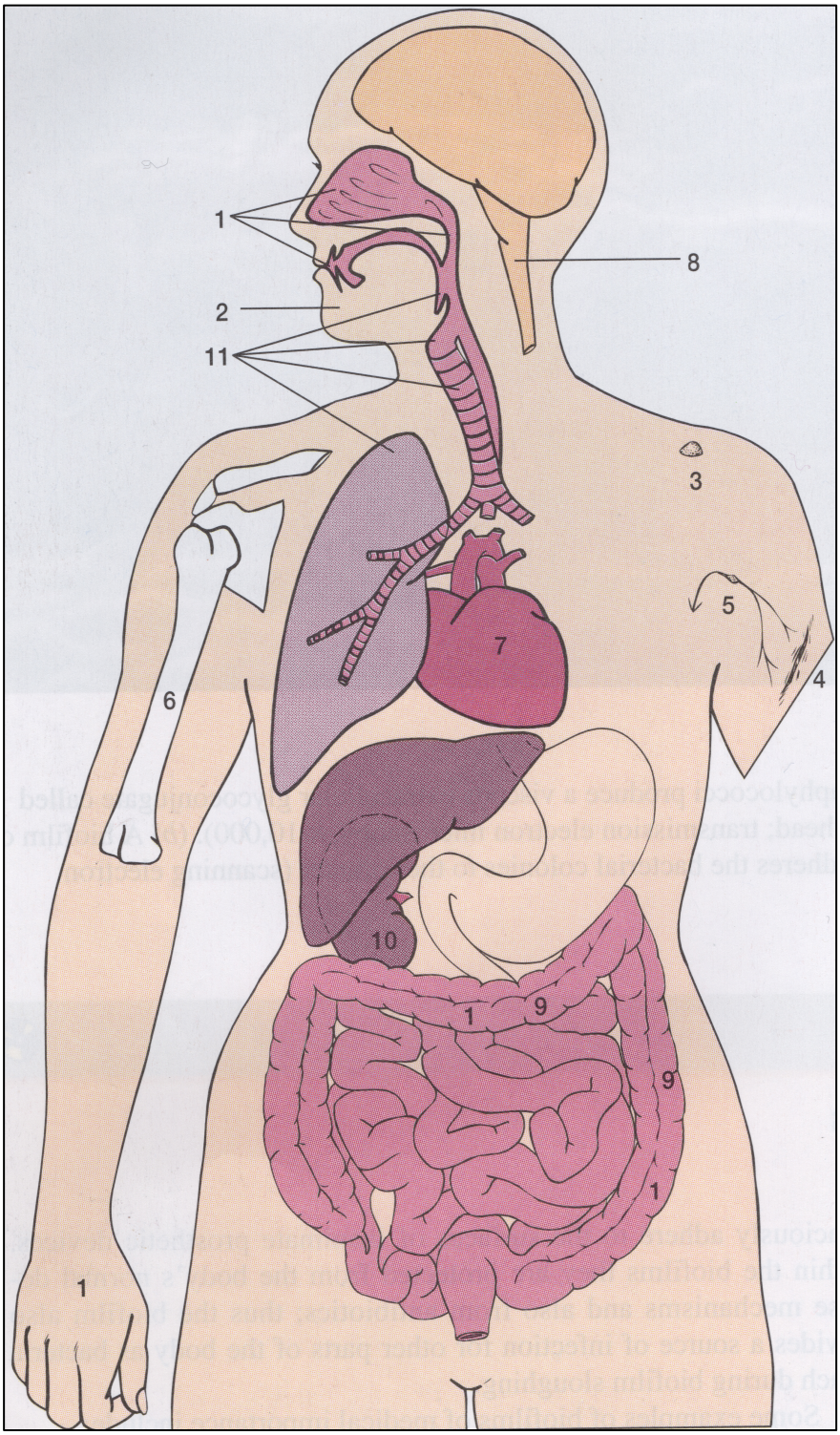

Figure 1.3: Sites for colonisation of* S. aureus *on humans and associated infections. 1. *Tissues where* S. aureus *is often found but does not cause disease,* ***2.*** *Pimples and impetigo,* ***3.*** *Boils and carbuncles on any surface area,* ***4.*** *Wound infection and abscesses,* ***5.*** *Spread to lymph nodes and to blood (septicaemia), resulting in widespread seeding,* ***6.*** *Osteomyelitis,* ***7.*** *Endocarditis,* ***8.*** *Meningitis,* ***9.*** *Enteritis and enterotoxin poisoning,* ***10.*** *Nephritis,* ***11.*** *Respiratory infections – pharyngitis, laryngitis, bronchitis and pneumonia (Prescott et al. 2002).*

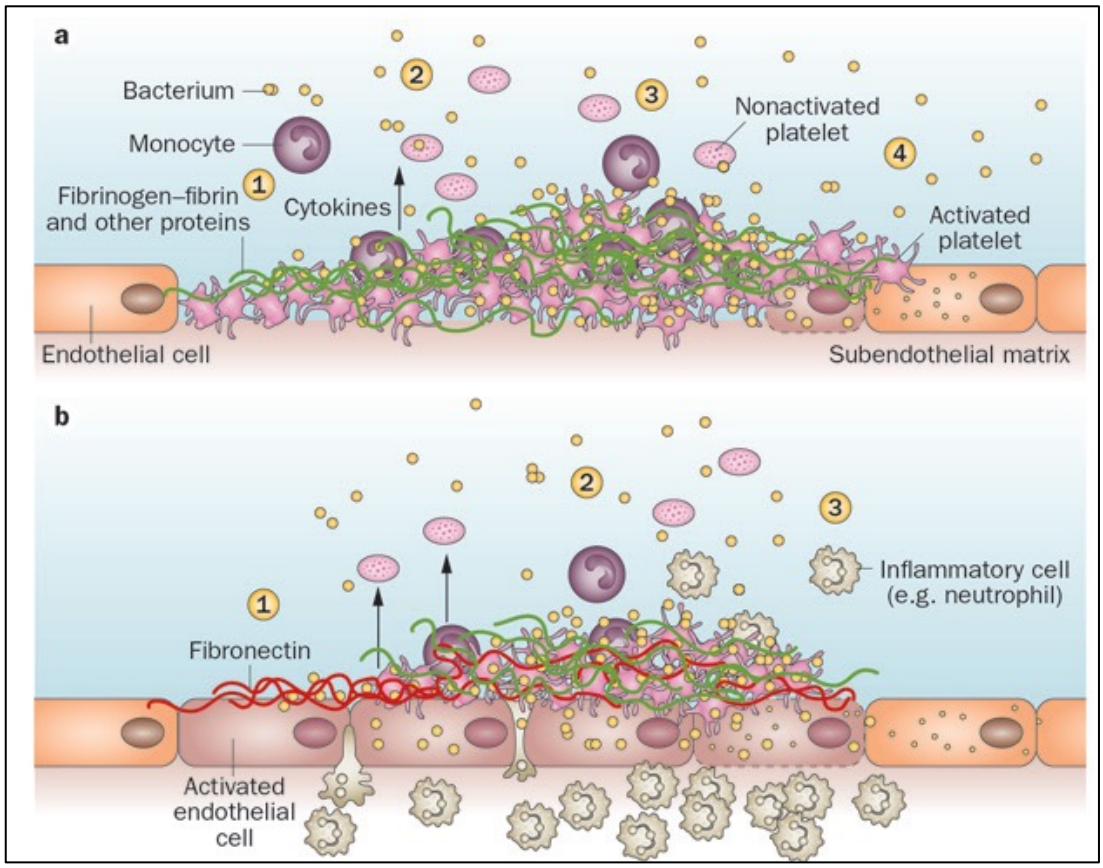

Figure 1.4: Mechanism of infective endocarditis: *a. Valve colonization as a consequence of mechanical injury. (1) Thrombus is formed consisting plasma proteins and platelets. (2) Bacteria bind to clot via platelets and plasma proteins and colonize it during transient bacteraemia. Adhered monocytes release tissue factor and cytokines. (3) More platelets are attracted and become activated, and the vegetation grows. (4) Endothelial cells are infected, and can be lysed by bacterial products, or bacteria can persist inside the cells. b. Valve colonization as a consequence of an inflammatory endothelial lesion. (1) Activated endothelial cells express integrins that promote the local deposition of fibronectin; bacteria such as* Staphylococcus aureus *adhere to this protein. (2) Bacteria are internalized, and endothelial cells release tissue factor and cytokines, causing blood clotting and promoting the extension of inflammation and vegetation formation. (3) Infected endothelial cells can be lysed by bacterial products, or bacteria can persist inside the cells (Werden et al. 2014).*

1.3. Platelets

Platelets are one of the three major blood cells and are smallest of all. The normal platelet count is 150,000-350,000/µl of blood (Pizzulli *et al*. 1998). Like other blood cells platelets are also produced in bone marrow from very large precursor cells called megakaryocytes (Deutsch & Tomer 2006). Functionally platelets are mainly involved in haemostasis but have a range of other less well-understood functions, such as inflammation (Lindemann *et al*. 2001), antimicrobial host defence (Cox *et al*. 2011), tumor growth and metastasis (Jurk *et al*. 2003), angiogenesis (Smyth *et al*. 2009) and immune regulation (von Hundelshausen & Weber 2007).

1.3.1. Platelet formation and clearance

Platelets are formed from megakaryocytes in bone marrow by the process of haematopoiesis (Deutsch & Tomer 2006). Although bone marrow is the major site for platelet production, small numbers are also produced in blood and the lungs (Zucker-Franklin & Philipp 2000). Megakaryocytes are developed from pluripotent hematopoietic progenitors. Before megakaryocytes can form platelets they become polyploid through endomytosis (repeated cycles of DNA replication without cell division) (Jackson 1990). Multiple sets of chromosomes result in amplification of functional genes thus leading to expression of functional platelet proteins and increase the cell size generating mature megakaryocyte (Vitrat *et al*. 1998). From the mature megakaryocyte, pro-platelets are generated, which are long thin cytoplasmic processes (Patel *et al*. 2005). Microtubules are responsible for elongation whereas actin based force is essential for pinching off to form platelets. Beside elongation, microtubules also

provide tracks for membranes, organelles and granules in proplatelets. The organelles move bidirectionally over microtubules until trapped in the pinched off platelet (Patel *et al*. 2005).

The life span of circulating platelets is 7 to 10 days after which they are cleared in the liver and spleen (Kaplan & Saba 1978). Many platelet surface proteins are glycosylated with a sialic acid cap (Crook 1991). Aging of platelets causes removal of the sialic acid cap leading to exposure of underlying sugar galactose. Ashwell-Morell receptors on liver hepatocytes and macrophages recognize desialylated platelets causing their clearance (Grozovsky *et al*. 2014).

1.3.2. Platelet Activation

The small size and discoid shape helps platelets to flow near the vessel edge in the circulating blood (Nesbitt *et al*. 2009). The position of platelets near the apical surface of the endothelium helps them to detect any vascular damage where they can adhere (Hartwig 2006).

Binding of platelets: Platelet adhesion is determined by rheological conditions that depend on the velocity of blood in the vessel and shear rate (Varga-Szabo *et al.* 2008). Shear rate occurs due to the shearing effects between the adjacent layers of fluid and is influenced by blood velocity and viscosity (Harris *et al*. 2015). The vessel wall creates higher shear rate on the blood flowing adjacent to it, thus higher shear rate exists at the wall surface and lower at the center (Lindemann *et al.* 2007; Varga-Szabo *et al.* 2008). Under high shear rates, the initial platelet adhesion with the ECM is mediated through the platelet receptor glycoproteins (GP)Ib and vWF, although this is the weak adhesion, but is important as it keeps platelets in close proximity with the endothelium

(Schneider *et al*. 2007). GPIb binds to immobilized but not the soluble vWf. Immobilization of vWf leads to its conformational change, like extension of its multimers due to shear stress that might expose the GPIb binding sites (Canobbio *et al.* 2004). During this phase, platelets roll over (Lindemann *et al.* 2007) the ECM and facilitate contact between the ECM protein collagen through an immunoglobulin superfamily receptor, GPVI (Nieswandt *et al.* 2009).

These interactions triggers intracellular signalling events coupled with platelet G-proteins and also induces the release of secondary mediator's; adenosine diphosphate (ADP) (Daniel *et al*. 1999) and thromboxane A2 (TXA2) (Nieswandt *et al*. 2001a; Liu *et al*. 2005). These shift low affinity platelet integrins towards their high affinity state and cause firm adhesion of platelets to exposed ECM proteins (Nieswandt *et al*. 2009). Secreted proteins from activated platelets recruit more platelets at the site of damage causing their activation and adhesion resulting in thrombus formation (Varga-Szabo *et al.* 2008; Nieswandt *et al.* 2009).

Spreading of Platelets: Binding to exposed ECM surface molecules or interaction with secreted agonists triggers changes in platelets, one of which is characteristic shape change (Lee *et al*. 2012). On contact, a platelet loses its discoid shape and becomes rounded or spheroid shaped which is followed by formation of finger like projections grown from the cell periphery (Kuwahara *et al*. 2002). Later, the platelet flattens over the surface and broad lamellae are extended, squeezing the cell organelles into the center giving it a fried egg like appearance (Lee *et al*. 2012). Finally, the dynamic phase of membrane motility occurs in which the membrane is disarranged and retracted inwards (Lee *et al*.

2012) forming filopods extended from cell center to cell periphery. Then the platelet completely spreads occupying significantly larger area compared to the resting form (Zhi *et al*. 2015a).

Transition of platelet shape change from discoid to sphere is induced by an increase in the cytosolic calcium levels. Intracellular calcium binds with gelsolin causing its conformational change. With this new conformation, gelsolin binds to actin, which causes splitting of actin filament resulting in disc to sphere shape change (Lind *et al*. 1982). Agonist stimulus to platelets also causes activation of G-proteins that activate Rho kinases and LIM kinases which phosphorylates myosin light chain kinases and cofilin (Offermanns 2006), facilitating reorganization of actin cytoskeleton forming filopodia and lamillopodia (Paul *et al*. 1999).

1.3.4. Granule Secretion

A platelet contains three types of granules – α, dense and lysosome, which are secreted on activation (Flaumenhaft 2003). Platelet activation causes the granules to centralize and may undergo homotypic fusion i.e. fuse with each other prior to secretion (Reed *et al*. 2000). The mechanism of secretion involves fusion of granules with platelet open canalicular system (OCS) (Flaumenhaft 2003). The OCS is the tunneling invagination of platelet plasma membrane and an elaborate channel system involved in pathway transport of substances into the cell and channels for discharge of granules (Escolar & White 1991).

α- granules are the most abundant of all platelet granules and are unique to platelets (Harrison & Cramer 1993). There are 50 to 80 granules/platelet ranging in the size from 200 to 500 nm that occupy 10% of platelet volume. On

activation, the granular membrane fuses to the platelet plasma membrane and granular contents are released (Blair & Flaumenhaft 2009). α- granules contain membrane bound proteins, some of which are already present on platelet plasma membrane like integrins (αIIb, α6, β3), immunoglobulin family receptors (GPVI, Fc receptors, PECAM) and other receptors like CD36 and Glut-3 (Berger *et al*. 1996). Other membrane proteins specific for α-granules are P-selectin, fibrocystin-L and CD109 that are only expressed on the platelet surface after granule release (Berman *et al*. 1986). There are known to be more than 300 soluble proteins in α- granules, most of which are already present in plasma. These soluble proteins have diverse functions in blood coagulation (coagulation factors, pathway inhibitors, tissue factors), cell adhesion (fibrinogen, vWf, thrombospondin) (Stenberg *et al*. 1984), inflammation, cell growth (epidermal growth factor, hepatocyte growth factor, insulin like growth factor) and host defence (microbicidal and immune response) (Blair & Flaumenhaft 2009).

Dense granules are subtypes of lysosome related organelles and are fewer and smaller than α- granules (Whiteheart 2011). These are exclusive to platelets and contain cations (Ca^{2+}, Mg^{2+}, K^{+}) (Ruiz *et al*. 2004), phosphates (polyphosphates, pyrophosphates), adenine nucleotides (ATP, ADP, GTP, UTP) (Fogelson & Wang 1996) and bioactive amines (serotonin, histamines) (Chatterjee & Anderson 1993). Membranes of dense granules contains proteins that are typically present in lysosome related organelle like LAMP-2 and CD63 (Israels *et al*. 1996).

Lysosomes in platelets contain enzymes that are present in other nucleated cells for protein degradation (cathaspin, elatase etc), carbohydrate

degradation (glucosidase, fucosidase, galactosidase etc) and phosphate ester cleaving enzymes – e.g. acid phosphatase is the most abundant (Ciferri *et al*. 2000). Lysosome contains LAMP-1, LAMP-2 and CD63 membrane proteins (Dell'angelica 2000).

1.3.5. Signalling in platelet activation:

Initial binding of platelets to vWf and collagen via GPIb-IX and GPVI respectively, induces intracellular signalling in platelets (Gardiner *et al.* 2010). GPVI is exclusively present on megakaryocytes and platelets (Moroi *et al.* 2004). GPVI consists of two immunoglobulins like domains in extracellular region and exists as a dimer on platelets forming two parallel grooves providing binding site for collagen (Horri *et al.* 2006). Initial low affinity interaction of collagen with GPVI is believed to cause clustering of the receptor (Jiang *et al.* 2015) and inside out signalling leading to activation of integrin α2β1 (Horii *et al.* 2006). Activated α2β1 is another collagen receptor (Emsley *et al.* 2000) that increases binding of GPVI to collagen (Nieswandt *et al*. 2001b). GPVI is non-covalently coupled with Fc receptor gamma chain (FcRγ), which plays part in signal transduction. Ligand binding to GPVI, causes activation of SFK's (specifically Lyn and Fyn) bound to its cytoplasmic domain, which then phosphorylate ITAM (Immune tyrosine based activation motif) domain of FcRγ (Watson *et al.* 2005). Syk kinase binds to the phosphorylated ITAM domains and causes phosphorylation of adaptor proteins (like LAT, SLP-76, Btk's, GADS), which leads to activation of PLCγ (Pasquet *et al.* 1999). Stimulation of GPIb-IX also activates SFK's and PI3 kinases causing calcium release and integrin activation (Liu *et al.* 2005). GPIb-IX is also involved

in the signal amplification in later stages of platelet activation which it does by association with FcRγ (Falati *et al.* 1999).

Together with exposed extracellular matrix and plasma proteins, various soluble factors also contribute in platelet activation (Rivera *et al.* 2009). Soluble platelet agonists are released from damaged cells, during coagulation, inflammation and from activated platelets which contribute in amplifying platelet response (Li *et al.* 2010). These molecules induce platelet response mainly via G- protein coupled receptors (GPCR's) which belong to family of seven transmembrane domain receptors that transmit signal through heterotrimeric G - proteins which are composed of α, β and γ subunits (Offermanns 2006). In a resting platelet α subunit exists in GDP bound state and on ligand binding it changes to GTP bound state and is released form the trimeric complex. α-GTP bound complex in cytoplasm interacts with downstream GPCR signalling cascade and causes platelet activation. β-γ complex is also involved in signalling which involves PI3Kγ as the downstream effector molecule (Li *et al.* 2010). Thrombin induced platelet activation is mediated via GPCR's called protease activated receptors (PAR's): PAR1 and PAR4 in humans and PAR3 and PAR4 in mice (Offermanns 2006).

Inside-out signalling

Platelets express integrins αIIbβ3 (fibrinogen receptor), α5β3 (vitronectin receptor), α2β1 (collagen receptor), α5β1 (fibronectin receptor), and α6β1 (laminin receptor) on their surface (Nieswandt *et al*. 2009). Integrin αIIbβ3 is the most abundant of all and is significant in platelet activation or aggregation. It is present in an inactive form (low affinity form) and on

stimulation of platelets, intracellular signalling causes its activation to a high affinity form (Gaul *et al*. 2015). The signalling leading to activation of platelet integrins is called inside out signalling (Mehrbod *et al*. 2013). Rap1 kinase an enzyme belonging to Ras family is important in causing activation of integrin by directly interacting with β3. Rap1 is present in a GDP bound state in the resting platelets and conversion to a GTP-bound form leads to its activation (Woulfe *et al*. 2002). At the initial stages of platelet activation Ca^{2+} and DAG-regulated guanine nucleotide exchange factor I (CalDAG-GEFI) are responsible for the conversion of GDP to GTP. Increase in the intracellular level of Ca^{2+} and DAG, in the initial phase of platelet activation leads to CalDAG-GEFI activation and in the later phase PKC/P2Y12 signalling activates Rap1 providing sustained platelet activation and stable thrombus formation (Li *et al*. 2010).

Outside-in signalling

Activated integrin αIIbβ3 can bind to fibrinogen (Litvinov *et al*. 2005), fibrin (Podolnikova *et al*. 2014) and VWF providing additional adhesion and causes aggregation of platelets (Mehrbod *et al*. 2013). Interaction of integrin αIIbβ3 with its ligands leads to intracellular signalling events (outside-in signalling) that trigger platelet spreading, granule secretion, stable adhesion, and clot retraction (Mehrbod *et al*. 2013). Ligands for αIIbβ3 cause clustering of the receptor, which is important for the signalling event to occur (Gaul *et al*. 2015). Proteins that bind directly to the cytoplasmic domains of αIIbβ3 include β3-endonexin, CIB1, talin, kindlin, myosin, Shc and the tyrosine kinases Src, Fyn and Syk (Zhi *et al*. 2015b). The change in conformation of the cytoplasmic domain of αIIbβ3 results in exposure of the tyrosine sites, which get phosphorylated and

provide the site for binding of signalling cascade proteins (Jenkins *et al*. 1998). Ligand binding to αIIbβ3 results in phosphorylation of cytoplasmic domain of FcγRIIa and inhibition of this phenomenon results in inactivation of amplification signalling during platelet activation (Boylan *et al.* 2008). Phosphorylation of FcγRIIa results in activation of Syk, which subsequently activates phospholipase C - γ2 (PLCγ2). Phospholipase C (PL-C) plays a role in release of calcium from the intracellular stores into the platelet cytosol. PL-C acts on membrane bound polyphosphoinositide $(PI)_{4,5}P_2$ converting it to diacyglycerol (DAG) and inositol 1,4,5 triphosphate (IP3). IP3 exists in soluble form in the cytosol that binds to receptors on dense membrane system in platelets and causes release of intracellular calcium whereas DAG causes activation of PKC, resulting in amplification of platelet activation signals (Baldassare *et al*. 1989). Outside-in signalling via αIIbβ3 results in complete platelet activation (Mehrbod *et al*. 2013).

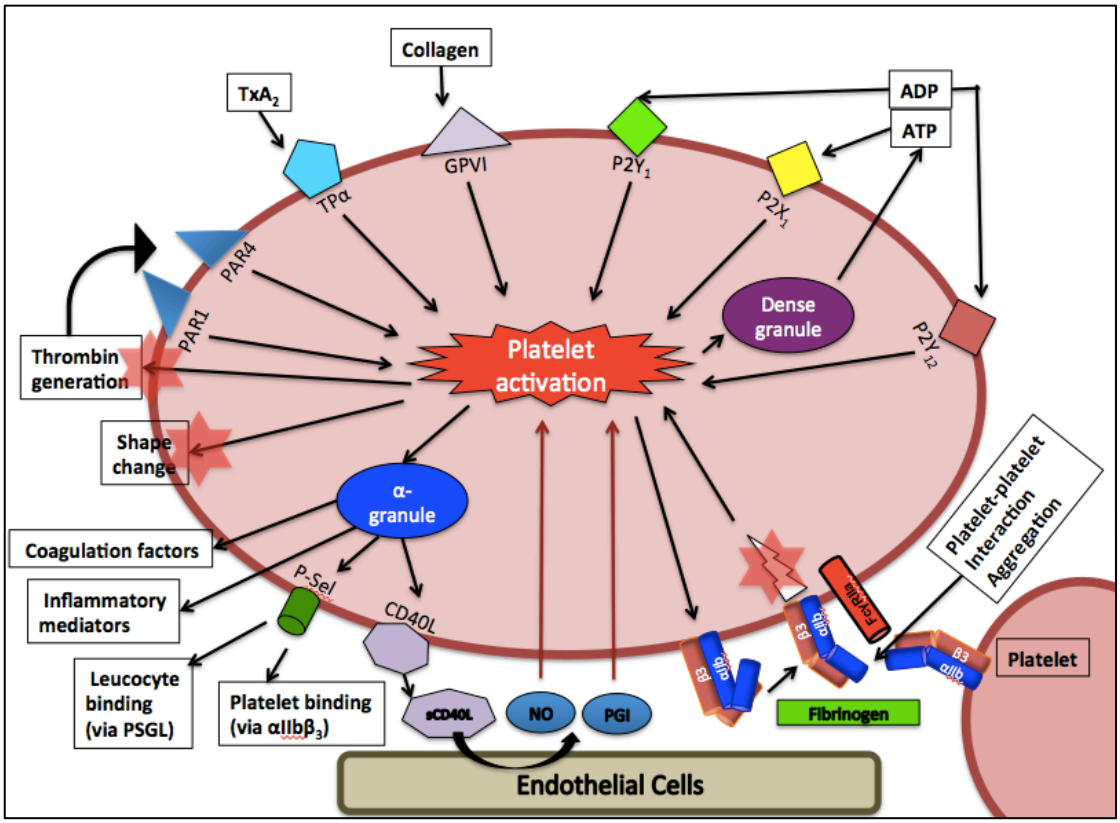

Figure 1.5: Platelet activation mechanism. *Platelet activation by different mechanisms leading to granule secretions, platelet shape change and integrin activation. Signalling events indicated by arrows (black) leads to integrin activation. Collagen activates platelets via GPVI. PAR receptors are activated by thrombin, thromboxin A2 (TxA_2) binds to TPα, ATP binds to $P2X_1$ and ADP to $P2Y_1$ and $P2Y_{12}$ causing amplification of platelet activation signaling. The lightning sign indicates outside in signalling event through activated integrin αIIbβ3 leading to complete platelet activation. Stars indicate the amplification response of activated platelets leading to its own and neighboring platelet activation. Red arrows indicate inhibition of platelet activation by NO and PGI_2 which are also released by endothelial cells on stimulation by soluble CD40L released from platelet surface.*

1.3.6. Inhibition of platelet function

In intact endothelium and vasculature, regulation of platelet function is essential in controlling hemostasis. In normal circulation platelet activation is inhibited by nitric oxide (NO) and prostacyclin (PGI2) (Mitchell *et al*. 2008).

Nitric oxide – is released by non-diseased blood vessels and intact endothelium (Tousoulis *et al*. 2012). It is antithrombotic and a vasodilator (Mitchell *et al*. 2008). In platelets NO binds to soluble Guanyle cyclase (sGC) resulting in its conformational change that increases its catalytic activity. Activated sGC increases level of cGMP that acts on cGMP dependent receptors, cGMP regulated phosphodiesterases (PDE) and activate cGMP dependent protein kinases (Gkaliagkousi *et al*. 2007). Increase in levels of cGMP decreases intracellular Ca^{2+} levels via cGMP dependent protein kinase G that inhibits entry of extracellular Ca^{2+} and its release from dense tubular system (Geiger *et al*. 1992). NO also inhibits αIIbβ3 by directly regulating phosphorylation of β3 (Oberprieler *et al*. 2007). P-selectin expression is affected by nitric oxide via inhibition of PKC (Murohara *et al*. 1995). Nitric oxide is also released upon platelet activation that is seen to be responsible for auto-regulation of enhanced platelet function during thrombus formation. Platelets regulate the effect of nitric oxide by desensitization of NO/cGMP system and producing reactive oxygen species (ROS) through glutathione cycle and lypooxygenase pathway (Bellamy *et al*. 2000; Friebe & Koesling 2003).

Prostaglandin (PGI2) – is a lipid derived from C-20 unsaturated fatty acid archidonic acid and is synthesized by the enzyme prostacyclin synthase, which is predominantly present in endothelial and smooth muscle cells

(Gryglewski *et al*. 1978). Platelets have PGI2 receptors – IP receptors that are members of the prostanoid family of G-protein coupled receptors (Klockenbusch *et al*. 1996). PGI2 on binding to IP receptors leads to series of signalling events resulting in increase of cAMP levels that causes activation of phospho kinase A (PKA) (Raslan & Naseem 2015). Activated PKA phosphorylates and activates myosin light chain kinases (MLCK), platelet inositol 1,4,5-triphosphate receptor and VASP at Ser239 (Aszodi *et al*. 1999). Activated MLCK phosphorylates myosin decreasing platelet contractile activity and inhibit Rho kinases thus subsequently inhibiting granule secretion (Aburima *et al*. 2013). The balance between TXA2 and PGI2 is essential for normal thrombotic function. TXA2 is a platelet agonist and vasoconstrictor and PGI2 counter acts these effects. The PGI2 receptor and TXA2 receptor can from a heterodimer. This heterodimerization has shown to increase the cAMP levels and lead to platelet inhibition (Gryglewski *et al*. 1978).

1.4. Platelets in the Immune Response

Platelets, mainly due their abundance in the blood stream, are the first cells to come into contact with a pathogen entering the blood (Shiraki *et al*. 2004). Along with the hemostatic function, platelets are also known to play a role in the immune and inflammatory responses (Weyrich *et al.* 2004). Various surface molecules on the platelet surface play important functions in its role in the immune system. P- selectin, a cell adhesion molecule expressed on activated platelets and endothelial cells, is important in the initial recruitment of leukocytes in immune or inflammatory responses (Palabrica *et al*. 1992). P-selectin binds to the P- selectin binding glycoprotein ligand 1 (PSGL1) and this reversible interaction is important in initial tethering, rolling and subsequent

firm arrest of the platelets (Frenette *et al.* 1995). Thus PSGL1 bearing immune cells can also bind to the platelets and thus facilitate in formation of platelet/leukocyte complexes and, vice versa, supporting leukocyte rolling and arrest on surface-adherent platelets (Cerletti *et al.* 2010; Hara *et al.* 2010).

Integrin αIIbβ3 is the most important surface molecule in the aggregation of platelets. Activated αIIbβ3 along with fibrinogen and von Willebrand factor can interact with CD40L triggering outside-in signaling (André *et al*. 2002; Prasad *et al*. 2003). Activated platelets are known to up-regulate the concentration of CD40L which interacts with endothelial cells via CD40 leading to increase in the concentration of adhesive molecules on their surface and consequently increase recruitment of immune cells (Dickfeld *et al.* 2001; May *et al.* 2002). CD40 is also present on monocytes (Alderson *et al*. 1993), dendritic cells (Ma *et al.* 2010), and B cells (Lee *et al*. 2003) and contributes to the development of the acquired immune response.

Activated platelets also secrete soluble immune mediators. The first of which to be identified was chemokine platelet factor 4 (PF4). PF4 is stored in α granules and in presence of TNFα it induces exocytosis and firm neutrophil adhesion to the endothelium (Petersen *et al.* 2014). PF4 interacts with other immune modulators and affects their function e.g. it binds to RANTES (Chemokine ligand 5) and promotes the monocyte adherence (Hundelshausen *et al.* 2005). Thrombin induced platelet activation releases RANTES which is necessary for P-selectin dependent monocyte adhesion to the endothelium (Schober *et al.* 2002; Hundelshausen *et al.* 2001).

Interleukin 1 (IL1) is an important immune and inflammatory mediator secreted by platelets (Loppnow *et al.* 1998). Platelets not only contain pre formed IL1 (Loppnow *et al.* 1998; Sedlmayr *et al.* 1995) but also show sustained synthesis, controlled at the translational level, by activation of platelet integrins (Lindemann *et al*. 2001). Thrombocidins, are platelet derived antimicrobial proteins, which are stored in α granules and secreted upon thrombin stimulation (Krijgsveld *et al.* 2000). They are bactericidal against *Bacillus subtilis*, *Escherichia coli*, *S. aureus*, and *Lactococcus lactis* and fungicidal for *Cryptococcus neoformans* (Krijgsveld 2000; Kwakman *et al*. 2011).

1.4.1. Toll like receptors (TLR's)

Direct evidence of the involvement of platelets in the immune system comes through the presence of toll like receptors. Platelets are known to posses TLR1, TLR2, TLR4, TLR6, TLR8, and TLR9 (Blair *et al*. 2009; Shiraki *et al*. 2004; F Cognasse *et al*. 2005). On activation by a physiological agonist (thrombin), expression of TLR-2 and TLR-4 is unchanged; however TLR-9 is increased suggesting that TLR-9 may be transported from intracellular compartments (Aslam *et al*. 2006). The exact function of TLRs on platelets is unclear but they are believed to play a role in their participation in immune and inflammatory responses. Activated platelets release a variety of prothrombotic, proinflammatory, and antimicrobial mediators (Weyrich *et al.* 2004) and play a role in endotoxin induced inflammatory responses, during which they modulates *in vivo* TNF α production, which is mediated through TLR-4 (Aslam *et al.* 2006; Stark *et al.* 2012). Platelet TLR-9 was found to be the receptor of carboxy (alkylpyrrole) protein adducts (CAPs) (Panigrahi *et al.* 2012) the end products of

phospholipid oxidation (Malinin *et al.* 2011) which links oxidative stress, innate immunity, and thrombosis. Platelet TLR-2 has a role in platelet- neutrophil aggregate formation in a P-selectin dependent manner (Blair *et al*. 2009). Also, platelets aggregate and adhere to collagen more readily on treatment with a known TLR-2 ligand Pam3CSK4 (Blair *et al*. 2009). TLR-2 stimulation has been shown to activate the phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway in platelets and induce secretion of proinflammatory mediators (Blair *et al*. 2009).

1.4.2: CD36

CD36 is known to be involved in metabolism, angiogenesis and immunity (Silverstein & Febbraio 2009). It is important in uptake of parasitized erythrocytes by macrophages during malaria infection (Ren 2012). Macrophage CD36 is attributed to many immune and inflammatory functions. It is speculated to be involved in inflammatory signalling cascade by interacting with other receptors such as TLRs. Macrophage CD36-dependent signalling is involved in the pro-inflammatory effects of internalizing β-amyloid (Moore *et al.* 2002), oxLDL (Maxeiner *et al.* 1998), and bacteria (Janabi *et al.* 2000). CD36 has been shown to associate with TLR2 in innate sensing and inducing inflammatory cytokines in response to TLR2 agonists such as whole bacteria like *S. aureus* (Stuart *et al.* 2005) and bacterial ligands (Triantafilou *et al.* 2006).

Microglia cell CD36 on β-amyloid uptake also boosts pro-inflammatory response and may contribute to the pathogenesis of Alzheimer's disease (Silverstein & Febbraio 2009). CD36 recognition of oxLDL and β-amyloid triggers assembly of a heterotrimeric complex composed of CD36, TLR4 and TLR6,

leading to the induction of pro-inflammatory mediators implicated in the harmful effects of oxLDL and amyloid-β in vivo (Stewart *et al.* 2010). TLR2 also requires CD36 for inflammatory signalling (Silverstein & Febbraio 2009). CD36 is necessary for the component of ischemic brain injury attributable to the inflammatory response triggered by TLR2/1 activation (Abe *et al.* 2010). The inflammatory response in brain induced by TLR2/1 activation, but not TLR2/6 or TLR4 activation, is suppressed in CD36-null mice (Abe *et al.* 2010). In contrast to brain inflammation, in systemic inflammation CD36 is involved in TLR2/6 activation, but not TLR2/1 activation (Abe *et al.* 2010; Triantafilou *et al.* 2006; Hoebe *et al.* 2005).

Role of CD36 in Oxidised LDL induced platelet activation is known (Magwenzi *et al.* 2015) but way in which it responds to other diacylglycerides moieties (specifically bacterial) is not yet understood. Significance of CD36 in immune and inflammatory response and its presence on platelets (Cserti-Gazdewich *et al.* 2009) makes it one of the important receptor to understand the role of platelets in immune response.

1.5. *S. aureus*–Platelet Interaction

The *S. aureus*–platelet interaction is a complicated process involving multiple receptors on both cells. von Willebrand factor (vWF) is the major glycoprotein which is necessary for the adhesion of platelets to the damaged vessel wall. Platelets do not have an affinity for the circulating vWF but can bind to immobilized vWF (Reininger *et al*. 2006). *S. aureus* 42kDa surface protein SpA which is made up of five domains each having IgG Fc region binding property (Fosteret *et al.* 1998; Graille *et al.* 2000) can also bind to von Willebrand factor

(Hartleib *et al.* 2000), which may be responsible for the initial contribution in binding to the platelets (Pawar *et al.* 2004). *S. aureus* can bind both soluble and immobilized vWF, which may contribute to entrapment of the bacteria in platelet plug (Pawar *et al*. 2004). Thus binding of SpA to GpIb-IX via a vWf bridge (Pawar *et al.* 2004; Zaverio *et al.* 2002) increases the contact time between platelet and bacteria facilitating the stable interaction between other surface proteins (Pawar *et al*. 2004).

Two fibrinogen (Fg) binding proteins (ClfA and ClfB) are expressed by *S. aureus*. Both proteins bind Fg at different domains. Fg is a large protein made from two sets of three polypeptide chains- Aα, Bβ and γ and thus is known as dimer of trimers (Mosesson *et al.* 2001). ClfA binds to the C-terminal of the γ-chain (McDevitt *et al*. 1997) and ClfB to the Aα and Bβ- chains (Ní Eidhin *et al*. 1998). In ClfA, the C- terminus of its A-chain binds to Fg and amino acids Glu^{526} and Val^{527} were found to be involved in the interaction (Hartford *et al.* 2001). Structural analysis of ClfA shows the presence of a Ca^{2+} binding EF hand motif and binding of Ca^{2+} causes a conformation change in ClfA, leading to a loss in its ability to bind Fg at the concentration of 1-10 mM Ca^{2+} (O'Connell *et al*. 1998). *In vivo,* Ca^{2+} is maintained between 1.0 and 1.3 mM (widely varying in extracellular spaces) and can inhibit the binding of ClfA to soluble Fg, thus allowing the bacteria, under the right conditions, to adhere to solid-phase fibrinogen or fibrin clots (O'Connell *et al*. 1998). Also, it has been speculated that Ca^{2+} dependent regulation of ClfA binding to Fg may aid bacteria to detach from the initial vegetation in order to proliferate (O'Connell *et al*. 1998). The interaction of ClfB with Fg is also through its A domain which is composed of sub domains: N1 which is elongated, N2, N3 and N23- which are globular (Perkins *et al*. 2001).

Cleavage of the N1 sub domain changes the ability of ClfB to bind the Fg. During the stationary phase of its growth *S. aureus* expresses the metalloprotease aureolysin which cleaves the N1 subdomain of ClfB (McAleese *et al.* 2001), changing the ability of ClfB to bind the Fg. The proportion of truncated ClfB increases as the *S. aureus* culture grows and the Fg – binding ability of ClfB is substantially reduced in stationary phase cells (McAleese *et al.* 2001) thus it shows the regulated ClfB binding (Perkins *et al.* 2001; Clarke *et al.* 2006).

By studying mutants of *S. aureus* that lacked one or more surface proteins and by expressing such surface protein individually on the surface of the surrogate Gram-positive host bacterium *Lactococcus lactis*, clumping factors (Clf) A and B were seen to cause activation of human platelets in plasma, of which ClfA is the more potent (L. O'Brien *et al*. 2002). Platelet activation through ClfA requires the presence of ClfA specific IgG and can occur with or without the fibrinogen bridge (Loughman *et al*. 2005). Fibrinogen-dependent platelet activation requires binding of ClfA with fibrinogen and the presence of IgG which interacts with platelet Fc receptor FcγRIIa on the platelets. Fibrinogen-independent platelet activation requires binding of IgG to ClfA and assembly of complement proteins, interacting with FcγRIIa and a complement receptor respectively on the platelet surface (Loughman *et al*. 2005).

Fibronectin-binding proteins, FnBPA and FnBPB are major platelet activating factors on the surface of *S. aureus* from the exponential phase of growth (Fitzgerald *et al.* 2006). FnBPA and FnBPB are structurally identical, being composed of four (A, B, C and D) domains consisting two and four repeating sequences of B and D domain respectively (Foster *et al.* 1998; Penkett

et al. 1998; Signas *et al.* 1989). FnBPA ligand binding D- domain lacks folded secondary structure and can bind to multiple sites on N-terminal of fibronectin (Foster *et al.* 1998) whereas its A- domain can bind with fibrinogen (Fitzgerald, *et al.* 2006; Foster *et al.* 1998). Thus FnBPA can bind to platelets via fibronectin and fibrinogen bridges that bind to the low affinity form of αIIbβ3 on platelets (Fitzgerald *et al.* 2006). Also another way of FnBPA interaction is through IgG reactive to FnBPA D-domain and A- domain, which engages with platelet Fc receptor FcγRIIa. These interactions of FnBPA with platelets led to platelet activation (Fitzgerald *et al.* 2006).

Another surface protein on the *S. aureus* surface, which is expressed under low iron conditions (typically a marker of *in vivo* conditions), the iron regulated surface determinant (Isd) proteins. One of these: IsdB can directly interact with the platelet integrin GPIIb/IIIa (Miajlovic *et al*. 2010), triggering their activation. Serine-aspartate repeat proteins (Sdr) have structural similarity with ClfA and ClfB (Josefsson *et al*. 1998). SdrE activates the platelets with longer aggregation lag time than that observed for other adhesions (L. O'Brien *et al*. 2002). Longer lag time indicates that the interaction of SdrE with its platelet receptor is relatively weak and requires a longer period of time to trigger the aggregation process (L. O'Brien *et al*. 2002). SraP is a glycoprotein located on the surface of *S. aureus*, which prevalent in clinical isolates and is associated virulence factor in infective endocarditis (Siboo *et al.* 2005). It mediates the direct and rapid binding of *S. aureus* to platelets (Siboo *et al.* 2005; Clarke *et al.* 2006).

S. aureus secreted α-toxin activates platelets either directly (Hugo *et al.* 1988; Schubert *et al.* 2011) or indirectly (Bayer *et al*. 1997). In human platelets, α-toxin is thought to stimulate activation by production of a prothrombinase complex on the platelet surface, which converts prothrombin to thrombin (Hemker *et al.* 1967), a potent inducer of activation in platelets (Vu *et al.* 1991). This occurs via two mechanisms. Firstly the attack of α-toxin causes the release of factor five (FV) stored within the α- granules of human platelets, which are released upon platelet stimulation by agonists such as thrombin and collagen. FV is then converted to factor five a (FVa) by thrombin. Both FV and FV(a) promote the assembly of a prothrombinase complex on the cell surface (Arvand *et al.* 1990). Secondly α-toxin directly acts by producing lesions on the cell surface, which produces an influx of calcium ions. These lesions alter the cell surface promoting the production of the prothrombinase complex in an exocytosis independent manner (Arvand *et al.* 1990). α-toxin also stimulates the arterial endothelial cells to synthesize the Platelet Activating Factors (PAF) (Bayer *et al*. 1997) thus promoting platelet activation. Other secreted products of *S. aureus* contributing to platelet activation are the two prothrombin activating molecules, staphylocoagulase and von Willebrand factor-binding protein (vWbp), which combine with prothrombin and form the staphylothrombin complex. The staphylothrombin complex has the ability to convert fibrinogen to fibrin and contribute to platelet activation (Vanassche *et al.* 2011).

In addition to activators of platelets, multiple *S. aureus* components have the ability to inhibit them. *S. aureus* LTA interacts with platelet PafR receptor causing increase in cAMP level thus resulting in inhibition of platelet activation (Waller *et al*. 2013). Inhibition of platelet function by the *S. aureus* LTA is

attributed to its ability to evade the immune system as this interaction also inhibits platelet-dependent inflammatory mediator release and platelet-monocyte aggregation (Wu *et al*. 2011). *S. aureus* also produces and secretes extracellular fibrinogen binding protein (Efb) which can inhibit platelet activation (Shannon *et al.* 2004). Efb can bind platelets directly or through fibrinogen. Both of these interactions are proposed to inhibit platelet activation and thus play vital role in delayed wound healing by *S. aureus* (Shannon *et al.* 2004).

1.5.1. FcγRIIa in *S. aureus* mediated platelet activation.

The FcγRII receptors are key activating and inhibitory effectors of the IgG-mediated immune functions (Ramsland *et al.* 2011). FcγRIIa in platelets is significant in amplification of outside in signalling occurring via αIIbβ3 (Boylan *et al.* 2008). It plays significant role in bacteria mediated platelet activation in synergism with integrin αIIbβ3 (Arman *et al.* 2014). Direct cross linking of FcγRIIa receptor leads to phosphorylation of tyrosine residues in its immunoreceptor tyrosine-based activation motif (ITAM) initiating platelet activation signalling events causing instant aggregation and secretion response (Arman *et al.* 2014; Ezumi *et al.* 1998; Marshall *et al.* 2004). Whereas in bacteria mediated platelet aggregation there is delay in the platelet aggregation response (Fitzgerald *et al.* 2006). Platelet activation on direct cross linking of FcγRIIa is independent of integrin αIIbβ3 engagement, but in the case of bacteria mediated platelet activation, phosphorylation of FcγRIIa is dependent on integrin αIIbβ3 engagement (Arman *et al.* 2014). Binding of IgG against bacterial antigen to FcγRIIa does not lead to sufficient stimulus to cause platelet activation, in

addition to that it requires feedback signal from engagement with αIIbβ3 and granule release (Arman *et al.* 2014). In the case of *S. aureus* it might occur on binding of ClfA/B with αIIbβ3 via fibrinogen bridge (McDevitt *et al*. 1997; Ní Eidhin *et al*. 1998). The engagement of αIIbβ3 and FcγRIIa causes weak signalling response that leads to release of granules that reinforces the activation signalling resulting in platelet activation and aggregation (Arman *et al.* 2014). Thus under arterial shear condition interaction between IgG and FcγRIIa along with engagement of integrin αIIbβ3 and granule release is thought to be of significance in *S. aureus* mediated infective endocarditis (Kerrigan *et al.* 2007, Krauel *et al.* 2014).

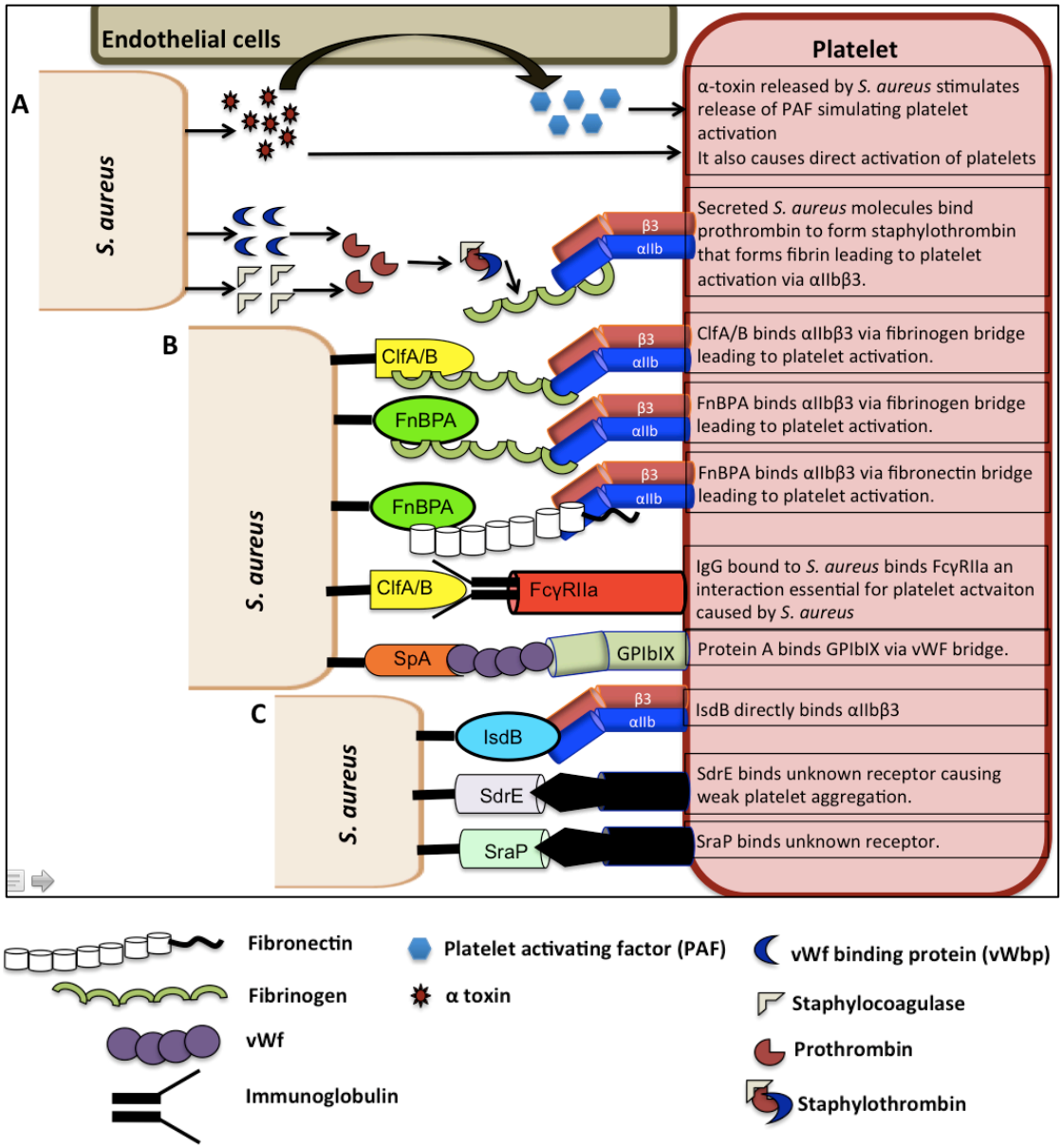

Figure 1.6: Interactions between* S. aureus *and platelets. A. *Platelet activation is induced by toxins and proteins released by* S. aureus*. **B.** Interactions between surface proteins on* S. aureus *with platelet surface receptors via plasma protein bridges. **C.** Direct interaction of* S. aureus *surface proteins with platelet receptors.*

1.6. *S. aureus* Lipoproteins

Bacterial lipoproteins are membrane-bound peptides bearing a covalently linked lipid moiety to a N-terminal cysteine residue which allows them to anchor to the membrane (Nakayama *et al.* 2012). Bacterial lipoproteins have been attributed with many functions like sensing (chemoreception) and signalling (initiate sporulation), protein secretion, antibiotic resistance, adhesion and uptake of many nutrients and ions (Sutcliffe *et al.* 1995). They have also been shown to play roles in virulence by supporting colonization, invasion, evasion of host defence and immune modulation (Kovacs-Simon *et al.* 2011; Nakayama *et al.* 2012). Bacterial lipoproteins play roles in induction of macrophages (Brightbill *et al*. 1999), dendritic cell maturation (Hertz *et al*. 2001), cytokine production (Kreutz *et al.* 1997) and activation of B- Lymphocytes (Bessler *et al*. 1985). It is clearly evident through a number of studies that the bacterial lipoproteins trigger intracellular signalling via toll like receptor-2 (TLR-2) on host cells (Aliprantis *et al.* 1999; Lien *et al.* 1999; Nakayama *et al.* 2012).

Using an *in silico* approach, approximately 50 putative lipoproteins have been identified in different strains of *S. aureus* (Sheldon *et al.* 2012), 35 of which can be associated with known or predicted function and many of them are part of ABC transporters and are involved in nutrient acquisition (Schmaler *et al.* 2009; Stoll *et al.* 2005). Several characterized staphylococcal lipoproteins, SirA, HtsA, SstD, FhuD1, FhuD2 and MntC are involved in ion acquisition (Sheldon *et al.* 2012); PrsA, the parvulin-type peptidyl-prolyl cis-trans isomerase involved in protein folding (Heikkinen *et al*. 2009); and OppA is the oligo peptide permease (Stoll *et al*. 2005). Lipoproteins are synthesized (Figure 1.6) as pre-prolipoproteins with an N-terminal signal sequence containing a conserved C-

terminal lipobox (sequence of conserved amino acids Leu- Ala – Gly – Cys (+1) – X (+2). A diacylglyceryl moiety is transferred to the invariant C-terminal cysteine by the lipoprotein diacylglyceryl transferase (Lgt) (Qi *et al.* 1995). The signal peptide of the prolipoprotein is cleaved by the lipoprotein signal peptidase (LspA) (Qi *et al.* 1995). While in Gram-negative bacteria lipoproteins are further modified at the diacylglyceryl-cysteine by N-acyltransferase (Lnt) (Kovacs-Simon *et al*. 2011), a homolog of Lnt has not been identified in *S. aureus* (Stoll *et al*. 2005), suggesting that *S. aureus* lipoproteins are diacylated.

S. aureus lipoproteins are essential for recognition of bacteria by the immune system (Bubeck Wardenburg *et al*. 2006). *S. aureus lgt* (lacking lipoproteins) is not efficiently recognized by immune cells (Stoll *et al*. 2005). *S. aureus* lipoproteins are capable of interacting with macrophages (Kim *et al*. 2015), human coronial epithelial cells (Li *et al*. 2008) and murine keratinocytes (Müller *et al*. 2010). TLR2 is found to be the major receptor for *S. aureus* lipoproteins (Schmaler *et al.* 2009) and interactions between them stimulate signaling and secretion events in the host cells (Kim *et al*. 2015; Li *et al*. 2008).

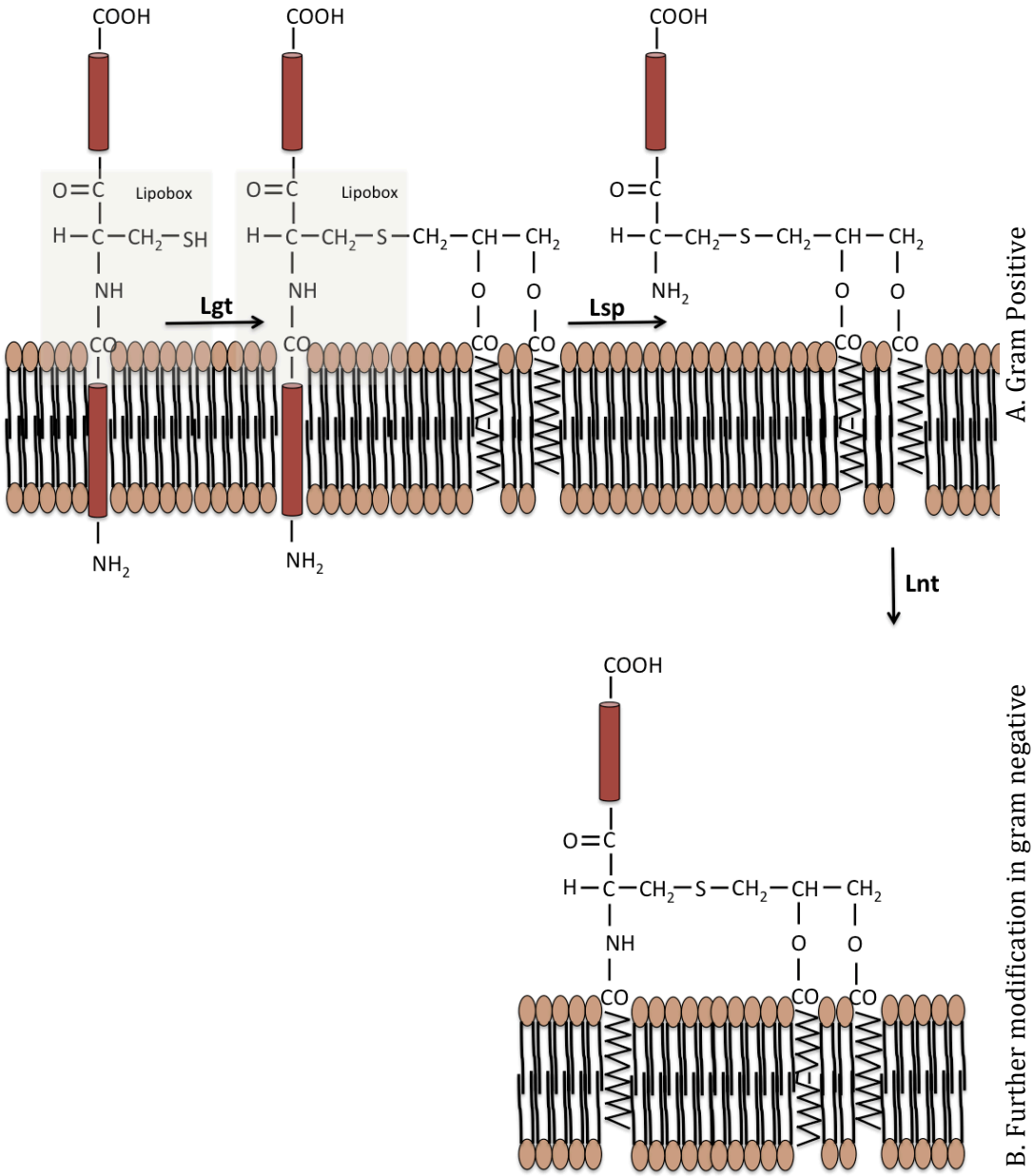

Figure 1.7: Biosynthesis of bacterial lipoproteins. *Two-step biosynthetic pathway in Gram-positive bacteria and three-step biosynthetic pathway in the case of Gram-negative bacteria. The precursor of lipoproteins is the preprolipoprotein, with an N-terminal signal peptide possessing a characteristic consensus sequence of the lipobox. During lipoprotein maturation, the thiol group of the invariant cysteine in the lipobox is modified by a diacylglyceryl moiety by lipoprotein diacylglyceryl transferase (Lgt), which serves as a membrane anchor. After lipidation, lipoprotein signal peptidase (Lsp) cleaves the signal peptide, leaving the cysteine as the new amino-terminal residue forming the mature lipoprotein in Gram-positive bacteria. In Gram-negative, the mature lipoprotein has an additional amide-linked fatty acid at the N-terminal cysteine residue attached by lipoprotein N-acyl transferase (Lnt).*

1.7. Aims of the project

With its varied strategies to survive in its host, *S. aureus* is a major pathogen causing diseases which range from moderate skin infections (Kintarak *et al.* 2004) to severe life threatening invasive conditions like endocarditis (Naber 2009). The ability of *S. aureus* to modulate platelet function is speculated to be significant in bacterial endocarditis (Ganesh *et al.* 2008) and delayed wound healing (Shannon & Flock 2004). Most of the *S. aureus* surface (L. O'Brien *et al.* 2002; Siboo *et al.* 2005; Fitzgerald, Loughman, *et al.* 2006) and secreted (Shannon & Flock 2004; Waller *et al.* 2013) molecules that can modulate platelet functions have been studied in an attempt to understand the pathology behind *S. aureus*-platelet interaction. But the role of *S. aureus* lipoproteins in this interaction is as yet unknown.

S. aureus lipoproteins are set of surface components essential for eradication of *S. aureus* infection by the host immune system (Bubeck Wardenburg *et al.* 2006). The ability of host immune cells to recognize *S. aureus* lipoproteins has made them prime target for vaccine discovery against *S. aureus* infections (Anderson *et al.* 2012). Platelets contribute the the immune and inflammatory response (von Hundelshausen & Weber 2007) and express receptors known for their immune function (Aslam *et al.* 2006). Toll like receptors and scavenger receptors are present on platelet surface that recognize lipoproteins (Valiyaveettil & Podrez 2009). Synthetic lipoproteins like Pam3CSK4 and plasma lipoproteins like oxidised LDL and HDL, modulate platelet function via these receptors (Fälker *et al*. 2014; Valiyaveettil *et al.* 2008; Nergiz-Unal *et al*. 2011).

Thus the aims of this project were, firstly to evaluate the ability of *S. aureus* lipoproteins to interact or bind with human platelets. During growth, *S. aureus* lipoproteins are released into extracellular milieu (Stoll *et al.* 2005) so the effect of extracted *S. aureus* lipoproteins on human platelets and their effect on thrombus formation in whole blood was examined. After establishing a platelet modulatory effect, platelet surface receptors and possible mechanism involved in the modulatory effect were studied.

Chapter 2
Materials and Methods

2.1. General Materials

2.1.1. Chemicals and Growth media

Chemicals were obtained from Sigma (Poole, UK), Acros organics (Geel, Belgium), Melford Lab. Ltd (Ipswich, UK) or Fisher Scientific (Loughborough, UK). Growth media were obtained from Oxoid (Basingstoke, UK).

2.1.2. Antibiotics

Antibiotics were obtained from Melford laboratories (Ipswich, UK).

2.1.3. Platelet agonists

Collagen was obtained from Nycomed (Munich, Germany), bovine thrombin from Sigma (Poole, UK) and cross-linked collagen-related peptide (CRP-XL) from Professor R. Farndale (University of Cambridge, UK) (Morton *et al*., 1995). Plasma oxidized LDL was provided by Dr. David Leake (University of Reading, UK).

2.1.4. SDS PAGE Gel electrophoresis

Acrylamide for protein SDS-PAGE was obtained from Severn biotech Ltd. (Kidderminster, UK). Gel electrophoresis equipment was from BioRad. Protein markers were obtained from Thermo Scientific.

2.1.5. Western blotting materials

PVDF membrane and protein markers were obtained from Thermo Scientific. Tween 20 was obtained from Fisher Scientific. BSA was obtained from Sigma. Semidry western blotting apparatus was obtained from BioRad. Enhanced

chemiluminescence (ECL) and Sigma fast alkaline phosphatase was obtained from Thermo Scientific Pierce and Sigma respectively.

2.1.6. Western blotting antibodies

Secondary HRP and alkaline phosphatase conjugated anti-mouse, anti-rabbit, anti-goat and anti-human antibodies were obtained from Sigma. Other antibodies are detailed in Table 2.13.

2.1.7. Co-precipitation assay materials

Streptavidin and protein A/G magnetic beads were obtained from GE healthcare life sciences. Biotin labeling to *S. aureus* lipoproteins was done using EZ-link Sulfo – NHS Biotin labeling kit from Thermo Scientific. Antibodies used are detailed in Table 2.12.

2.2. Preparation of bacteria and bacterial components:

2.2.1. Bacterial growth curve

S. aureus cells were inoculated into BHI medium and incubated at 37^{o}C for 16 hrs with shaking. OD_{600} of cultures was measured to confirm that the cultures were in post exponential phase and 1% of that culture was inoculated in BHI media and incubated at 37^{o}C in shaking. OD_{600} was taken at 30 min intervals and a graph of $LogOD_{600}$ against time was plotted.

2.2.2. *Staphylococcus aureus* transduction

Mutant *S. aureus* donor strains were grown in LB broth (Table 2.1) with antibiotics for 16 hrs at 37^{o}C. The culture was centrifuged at 3345 x g for 10 min and pellet was resuspended in 5 ml LB such that the optical density at 600 nm (OD_{600}) was 0.1 and was transferred to detergent free, sterile 100 ml flask.

Sterile phage buffer (100 ml) (Table 2.1) and 100 µl of stock lysate (phage – ϕ85) was added. The suspension was incubated for 10 min at 22°C, later for 4 hrs at 30°C and finally at 22°C until a clear lysate was formed which was filter sterilized and used for transduction. An *S. aureus* colony was inoculated in LK broth (Table 2.1) and incubated at 37°C for 16 hrs. The culture was centrifuged at 3345 x g for 10 min and the pellet was resuspended in 2 ml of LK broth. To three sterile universal tubes 500 µl of cell suspension was added. In the first two 1 ml and in the third 1.5 ml of LK broth with 10 mM $CaCl_2$ was added. To the first universal tube 500 µl and in second 100 µl of phage lysate was added. The third universal tube was kept as a control without any phage lysate in it. The universal tubes were incubated in a 37°C water bath for 25 min and then in a shaking incubator for 15 min. Later, the universals were kept on ice for 5 min after adding ice cold 0.02 M Sodium citrate. The suspension was centrifuged at 3345 x g for 10 min and the pellet resuspended in 1 ml 0.02 M Sodium citrate and left on ice for 30 min. Aliquots of 100 µl and 200 µl from each universal were spread on LKcit (2.94% of 0.1 M sodium citrate) plates with antibiotics at 1/33 dilution of Minimum Inhibitory Concentration (MIC). After incubation at 37°C for 90 min (or until shiny sheen appearance appeared) the plates were overlaid with 5 ml LK top agar containing respective antibiotics at 6X MIC, which were then incubated at 37°C until growth was usable (~48 hrs). The colonies were carefully picked by peeling back the top agar and streaked on to agar plates with respective antibiotics and incubated at 37°C overnight. A single colony from plates was then inoculated into LB broth containing antibiotics and growth was checked for confirmation of transduction (Novick 1967). Glycerol stocks of the mutant were prepared.

2.2.3. Bacteria Cell Envelope Extraction

S. aureus wild type and mutants were inoculated into chelex treated BHI medium and incubated at 37^{o}C for 16 hrs with shaking. Pre-warmed 100 ml chelex treated BHI medium was inoculated and grown until late-exponential phase (OD_{600}– 1) at 37^{o}C with shaking. Using these cultures 100ml of pre warmed chelex treated BHI medium was inoculated to a starting OD_{600} of 0.05 and incubated until OD_{600} reached 1. Cultures were centrifuged at 8000 x g for 5 min and resuspended in 1 ml cold TBS. Then on centrifugation at 8000 x g for 5 min the bacteria were resuspended in 1 ml cold extraction buffer solution (Table 2.4) and transferred to FastPrep tubes. Cells were disrupted at 2000 oscillations/min for 7 min and glass beads (in FastPrep tubes) were allowed to settle. Suspension was removed into universals and 5 ml of cold 50 mM Tris (pH-7.5) and 0.1 M NaCl solution was added. The samples were centrifuged at 209 x g for 10 min at 4^{o}C and supernatant was collected in new universals. Cold 50 mM Tris (pH-7.5) and 0.1 M NaCl solution was added and centrifuged at 3345 x g for 10 min at 4^{o}C. Pellets were resuspended in 1 ml cold 50 mM Tris (pH – 7.5) and transferred to eppendrof tubes. The tubes were centrifuged at 8000 x g for 10 min to obtain the native cell envelopes (Clarke *et al.* 2002). The isolated cell envelope were lyophilized and used for the assays.

2.2.4. Lipoprotein isolation (TX-114 phase partitioning method)

S. aureus grown in chelex treated BHI to the mid log phase was centrifuged and the pellet was resuspended in 4 ml 1 M NaCl. Cells were transferred to FastPrep tubes (MP Biomedical) for disruption at 2000 oscillations/min for 7 min. Glass beads were allowed to settle and the liquid

portion was transferred to a new tube. After centrifugation at 2000 x g for 10 min supernatant was collected in a new tube and again centrifuged at 10000 x g for 10 min. Supernatant was transferred to a new tube and Triton X-114 was added to a final concentration of 2% and incubated at 4^{o}C for 1 hr. For the phase separation the tubes were incubated at 37^{o}C for 10 min before centrifugation at 10000 x g for 10 min. The replacement of the aqueous phase with same volume of PBS was repeated three times. Excess methanol was added to precipitate the lipoproteins and centrifuged at 10000 x g for 10 min. The methanol wash was repeated twice and the precipitates were dialyzed in nano pure water at 4^{o}C and lyophilized (Asanuma *et al.* 2011).

2.3. Platelet preparation

2.3.1. Preparation of Platelet Rich Plasma (PRP)

Blood samples were obtained from healthy donors who had not taken any non-steroidal anti-inflammatory drugs for 10 days prior to sampling. This study was performed according to institutional guidelines and was approved by the Institutional Ethics Committee. Written consent was obtained from all of the donors. 45 ml blood in 5 ml of 4% sodium citrate was taken. Blood was centrifuged at 102 x g for 20 min at 20^{o}C. PRP was carefully pipetted out and used for platelet aggregation studies at a concentration of 4 x 10^{8} cells/ml.

2.3.2. Preparation of Washed Platelets

Washed platelets were prepared from 50 ml blood mixed with 7.5 ml of ACD (Acid Citrate Dextrose), which was then centrifuged at 102 x g for 20 min at 20^{o}C. PRP was removed and 10 µl of prostacyclin (PGI) (250 µg/ml) was added to it before centrifugation for 10 min at 1413 x g at 20^{o}C. The pellet was

resuspended in 150 µl of ACD and 1 ml Tyrodes buffer. Then 3 ml of ACD was added and final volume to 25 ml was made using Tyrodes buffer. PGI (10 µl) was added and the concentration of platelets was calculated using Coulter counter (Beckman Coulter, USA), for which 2 µl of platelets in 10 ml of isoton solution was used. Platelet suspension was then centrifuged for 10 min at 1413 x g at 20^{o}C and platelet pellet was resuspended in Tyrodes buffer such that concentration was 4x10^{8} cells/ml (Asselin *et al*. 1997).

2.3.3. Preparation of platelet lysates

Washed platelets (450 µl) were treated with the agonist or the *S. aureus* lipoprotein extract (50 µl) in aggregometry cuvettes. SDS PAGE sample loading buffer was added to arrest the platelet response. These samples were heated at 90^{o}C for 10 min then loaded on SDS PAGE gels and western blotted for desired proteins.

2.4. Platelet function assays

2.4.1. Platelet Aggregation

Platelet aggregation was studied using an Aggregometer (Chrono-Log). *S. aureus* wild type and mutant strains were inoculated into Chelex treated BHI and incubated at 37^{o}C for 16 hrs with shaking. These cultures (50 µl) were inoculated into 5ml Chelex BHI and grown at 37^{o}C with shaking until OD_{600} reached 3. Bacteria were washed three times in Tyrodes buffer and finally OD_{600} was adjusted to 1 in Tyrodes buffer which equals to concentration of 6 x 10^{8} bacteria/ml. Platelet poor plasma (PPP) was used as blank for PRP and Tyrodes buffer as blank for washed platelets. The blanks and 450 µl of platelets were added to the cuvettes and placed in the respective aggregometer chambers. Light

transmission was recorded for specific times after adding 50 µl of bacteria or cell envelope extracts to the platelets. Percentage aggregation was calculated from the amplitude obtained in the aggregation traces considering wild type as 100%. For aggregation assays with platelets receptor blocking, platelets were incubated with respective antibodies (Table 2.14) or their isotype controls. For looking at effect of *S. aureus* lipoproteins and synthetic lipoproteins (Table 2.14) platelets were preincubated with proteins in aggeregometry cuvettes prior to the addition of agonist.

2.4.2. Platelet – Bacteria binding assay

Poly-L-lysine, 1 mg/ml in tyrodes buffer (100 µl) was added into the well of a 96 well plate for 5 min then washed with tyrodes buffer and dried overnight. Washed human platelets were prepared as described above. Washed platelets were fixed by 0.8% formaldehyde at 37^{o}C for 30 mins. Fixed platelets were washed three times with tyrodes buffer using centrifugation at 1413 x g for 10 min at 20^{o}C. Each well was loaded with 100 µl of fixed platelets and the plate was incubated at 37^{o}C for 30 min. 1% casein w/v in tyrodes buffer was then added to wells and incubated for 1 hr. *S. aureus* wild type and mutant bacteria cultures were grown till mid log phase in BHI and were washed three times with tyrodes buffer using centrifugation at 209 x g for 10 min at 4^{o}C and the final OD_{600} was adjusted to 1. Bacteria were diluted 1:10 v/v in tyrodes buffer before adding 100 µl to each well of the platelet-coated plate. After incubation of plates at 4^{o}C for 2 hrs the wells were washed three times with tyrodes buffer to remove unbound bacteria. Bound bacteria were removed into 100 µl of tyrodes buffer. Serial dilutions were prepared and spread onto BHI plates, which were

incubated for 16 hrs at 37^{o}C. The number of bacteria was counted on each plate and percentage binding to platelets was calculated by subtracting platelet free controls and dividing by total bacteria added.

2.4.3. Flow-cytometry

Two sets of clean eppendorf tubes were prepared and labeled as EGTA, resting, agonist controls and agonist plus samples (*S. aureus* lipoproteins) at different concentration. HEPES buffer (Table 2.10) was added to tubes at a volume such that on addition of all solutions final volume reaches to 50 µl. GPRP and EGTA were added to appropriate tubes. *S. aureus* lipoproteins were added at an appropriate volume to obtain desired concentrations. Fluorescently tagged antibodies (Table 2.15) were added for desired proteins. Whole blood (human or mouse) was added and incubated for 20 min with mixing at regular intervals. For receptor blocking assay the relevant antibody (Table 2.13) was added to blood prior to the addition of *S. aureus* lipoproteins. The agonist was added at appropriate concentrations and incubated for 20 min at room temperature before 450 µl of 0.2% formaldehyde was added to stop the reaction. In FACS tubes with 450 µl of 0.2% formaldehyde, 50 µl of the sample was added in relevant tube and run through flowcytometer (BD Accuri™ C6 cytometer). During analysis the platelets were gated on the basis of size and 5000 events were measured for each sample. The median fluorescence was measured through appropriate filters (FITC conjugate – FL2 and PE conjugate – FL3) and represented after normalizing to the control as 100%.

2.4.4. Platelet spreading

Coverslips were placed in a 24 wells plate and coated with fibrinogen by adding 200 µl of 100 µg/ml fibrinogen then incubated at 37^{O}C for one hour. Each coverslip was washed two times with 200 µl of PBS and blocked by 1% BSA - PBS solution for one hour at room temperature then washed twice with PBS. Washed platelets were prepared at a concentration of 2 x 10^5 cells/ml and were incubated with different concentrations of lipoproteins. After incubation for 15 min at 37^{O}C, 200 µl of the platelet-lipoprotein mixture was added to the wells with fibrinogen coated coverslips and incubated for 30, 45 and 60 min time points at 37^{O}C before washing twice with tyrodes buffer. After washing coverslips, platelets were fixed with 4% PFA solution for 10 min at 37^{O}C and washed twice with PBS. Platelets were permeablised using 0.1% Triton-100 for 10 min at 37^{O}C and washed twice with 1X PBS. Staining was done by adding 200 µl of phelloidin 647 in each well at 5:200 dilution in 1%BSA - PBS solution for 20 min at room temperature in dark. After washing twice with PBS, coverslips were carefully removed from the wells and mounted on the clean slides using mounting oil. Slides were observed under confocal microscope and images were analyzed using ImageJ.

2.4.5. Calcium assay

Fura-2 was added to final concentration of 2 µM in PRP and was protected from light. After incubation at 30^{O}C for 1 hour by inverting every 15 min this mixture was centrifuged at 350 x g for 20 min and the platelet pellet was resuspended in tyrodes buffer at concentration of 4 X 10^8 platelets/ml. Platelets (80 µl) were loaded in black 96 well plate with different concentrations

of *S. aureus* membrane proteins and with nanopure water as a negative control and incubated for 10 min. The plate was incubated in NOVOstar plate reader (set at 37^{O}C). Fura-2 measurement protocol "$[Ca^{2+}]_I$AB" with settings to add 20 µl of agonist (thrombin) at 5X concentration and to record fura-2 fluorescence – excitation at 340 and 380 nm, emission at 510 nm for 2 mins 20 sec. Agonist was added after 10 sec to allow measurement of resting $[Ca^{2+}]_i$.

2.4.6. Thrombus Under Flow

Preparation of cellix slide: Collagen at 400 µg/ml was injected at one end (which was labeled to point out the end used to load the collagen) of the cellix capillary such that a quarter of capillary surface area was covered. Each slide was incubated for an hour at room temperature and 20 µl of 1% BSA was added from the opposite side of the capillary. On incubation for an hour at room temperature the capillaries were filled with 100 µl of tyrodes buffer from the same side as the collagen was added.

Thrombus Under flow assay: Whole human citrated blood was incubated with *S. aureus* membrane protein extracts for 20 min. After incubation, 3,3-dihexyloxacarbocyanine iodide (DIOC6) (100 µg/mL of whole blood) dye was added and perfused through collagen-coated (400 µg/mL) Vena8TMBiochip (Cellex, Dublin) at a shear rate of 20 dynes/cm^2. Z-stack images of forming thrombi were taken every 30 sec using a Nikon eclipse (TE2000-U) microscope and thrombus fluorescence intensity was analyzed using SlidebookTM5 software (Intelligent Imaging Innovations, Denver, USA).

2.5. Biochemical Analysis

2.5.1. SDS PAGE

All small SDS-PAGE gels were prepared using a Mini – Protein Electrophoresis System (BioRad, UK) and large SDS-PAGE gels using the Protein – II xi system (BioRad, UK). All buffers and gel solutions were prepared as described in Table 2.5. For electrophoresis small gels were run at 125 volts for 2 hrs and large gels at 200 volts for 7 hrs. Following electrophoresis gels were either used for staining or western blotting.

2.5.2. Coomassie staining

The gel was placed in Coomassie staining solution (Table 2.6) for 2 - 4 hrs on a rocker, until the gel was a uniform blue color. Gels were destained (Table 2.6) for 2 - 6 hrs on rocker until the background was clear and bands were visible.

2.5.4. Silver staining

The gel was incubated in fixing solution (Table 2.7) for 30 min (for small gel) or 1 hour (large gel) then washed three times with distilled water for 10 min and incubated in sensitizing solution (Table 2.7) for 30 min. After washing three times for 10 min with distilled water the gel was incubated in 2.5% silver nitrate solution for 20 min and again washed three times with distilled water for 2 min. Developing solution was added and changed immediately when it turned yellow. Staining was terminated once the bands were visible by adding stopping solution (Table 2.7). After staining the gels were washed three times with water for 5 min and preserved in 5% acetic acid solution.

2.5.5. Mass spectrometry

Protein samples to be analyzed by mass spectrometry were loaded on the SDS PAGE gel and silver stained. All the materials used during this process were washed with ethanol and care was taken during procedure to avoid keratin contamination. Desired bands were cut and placed in eppendorf tube to be sent for mass spectrometry analysis at Advanced Mass Spectrometry Facility, University of Birmingham. The analysis was reported to be done on Orbitrap Mass Spectrometer for protein identification using bottom up LC (liquid chromatography) MS/MS by ETD (Electron Transfer Determination) method.

2.5.6. String analysis of Mass spectrometry data

Mass spectrometry data was submitted to string database (http://string-db.org/) (Jensen *et al.* 2009) for identification of protein-protein interactions. For identification of network all the parameter like experimental repositories, computational prediction methods and public text collections were chosen. The protein interactions around CD36 were done manually by focusing around it from entire network and only protein nodes (connections) that connected with CD36 were chosen to be displayed (Figure 6.4).

2.5.7. Western blotting

Protein samples were mixed with SDS PAGE loading buffer and were heated at 90^{o}C for 10 min and loaded on to SDS PAGE gel and electrophoresed. Three filter papers were soaked in western blot transfer buffer (Table 2.8) and placed in semi dry blotter. PVDF membrane soaked in methanol was placed on to the filter papers and the gel soaked in western blot transfer buffer was placed on to the membrane. Another three filter papers soaked in western blot transfer

buffer were placed on to the gel creating a sandwich and a voltage of 15 for 2 hrs was applied for blotting. The membrane was blocked with 5% BSA solution overnight at 4^{o}C. After blocking a specific primary antibody was added and incubated as instructed by manufacturer. The membrane was then washed three times with TBS-T (Table 2.8) for 10 min each time and incubated with a secondary antibody (1:4000) for 1 hour at room temperature. After washing three times with TBS-T for 10 min, depending on the secondary antibody, the blot was developed using Sigma alkaline phosphatase or by ECL.

2.5.8. Co- precipitation assay

Washed platelets at 8 X 10^8 cells/ml were placed in aggregometry cuvettes and lysed by NP40 lysis buffer (Table 2.11) and immediately placed on ice. The lysed platelet suspension was centrifuged at 10000 x g for 2 min to remove cytoskeleton proteins. The supernatant was collected into fresh tubes and washed 12.5 µl streptavidin beads or protein A/G magnetic beads were added as a pre-clearing step to remove nonspecific binding. Beads were removed by a magnet and the platelet lysate suspension was collected into a fresh tube. Biotinylated *S. aureus* lipoproteins at concentration of 10 µg/ml or antibodies for the specific protein to be co-precipitated (Table 2.12) were added and tyrodes buffer was added as a negative control in a different tube. The mixture was incubated at 4^{O}C for an hour and 25 µl of washed streptavidin beads or protein A/G magnetic beads were added. After incubation with beads for an hour at 4^{O}C, beads were collected using a magnet and washed once with ice-cold NP40 lysis buffer and twice with PBS-T (0.025% Tween 20). To remove proteins bound to beads, 100 µl of SDS PAGE sample loading buffer was added. Samples were

heated at 90^{O}C for 10 min prior to loading on the gel and then silver stained or western blotted for looking at co-precipitated proteins.

2.6. Statistics

All the assays were repeated three times and on three or more donors. All results are presented as mean ± SEM. Students t-test or anova test were used for statistical analysis. A p-value of <0.05 was considered to be statistically significant.

Table 2.1: Growth media used in *S. aureus* transduction

LB broth (100 ml)		LK broth (100 ml)	
Tryptone	10 g	Tryptone	10 g
Yeast extract	5 g	Yeast extract	5 g
NaCl	10 g	KCL	10 g
pH	7.5	pH	7.5

Table 2.2: Buffers used in *S. aureus* transduction

Phage buffer	
$MgSO_4$	1 mM
$CaCl_2$	4 mM
Tris-HCl (pH-7.8)	50 mM
NaCl	100 mM
pH	7.8

Table 2.3: *S. aureus* strains and mutants used in study

Strain	Phenotype	Reference or Source
S. aureus SA113	Wild type	(Iordanescu and Surdeanu, 1976)
S. aureus SH1000	Wild type, *rsbU*$^+$ repaired	(Horsburgh *et al.*, 2002)
S. aureus SA113 *lgt*	*lgt:: ermB* Em^R	(Stoll *et al.*, 2005)
S. aureus SA113 *ΔltaS*	*ΔltaS*::Sp^R	(Peschel *et al.*, 1999)
S. aureus SA113 *ΔltaS lgt*	*ΔltaS lgt* ::Sp^R Em^R	Current Study

erythromycin resistant (Em^R), spectinomycin resistant (Sp^R)

Table 2.4: Buffers used in Bacteria Cell Envelope Extraction

Tris Buffered Saline (1000 mL)		**Extraction buffer solution**	
Tris	9.68 g	Tris (pH-7.5)	50 mM
NaCl	32 g	NaCl	0.1 M
KCl	0.2 g	PMSF	0.5 mM
Nanopure water	Added to make up final volume.	pH	7.5
pH	7.4		

Table 2.5: SDS PAGE Solutions

Separating Gel	10%	13%
3M Tris HCl (pH 8.8)	1.25 ml	1.25 ml
Nano pure water	5.377 ml	4.335ml
10% SDS (in nanopure water) (w/v)	100 µl	100 µl
Acrylamide/bis (30%)	3.33 ml	4.21ml
10% APS (in nanopure water) (w/v)	50 µl	50 µl
TEMED	10 µl	10 µl

Stacking Gel	4%
0.5M Tris HCl (pH 6.8)	1.25 ml
Nano pure water	2.918 ml
10% SDS (in nanopure water) (w/v)	100 µl
Acrylamide/bis (30%)	0.667 ml
10% APS (in nanopure water) (w/v)	50 µl
TEMED	15 µl

SDS Sample Buffer (10 ml)		SDS PAGE Running Buffer	
1M tris/HCl (pH 6.8)	2.5 ml	Tris base	124 mM
Glycerol	4 ml	Glycine	960 mM
SDS	1 g	SDS	17 mM
2-mercaptoethanol	2 ml		
0.1% Bromophenol blue	0.8 ml		
Nanopure water	Added to make up final volume.		

Table 2.6: Coomassie Staining Solutions

Coomassie Staining Solution		**Destain Solution**	
Coomassie Blue R-250	0.1%	Glacial Acetic Acid	10%
Glacial Acetic acid	10%	Methanol	45%
Methanol	40%	Nanopure water	45%
Nanopure water	49.9%		

Table 2.7: Sliver Staining Solutions

Fix Solution		**Sensitizing Solution (250 ml)**	
Glacial Acetic Acid	10%	Ethanol	75 ml
Methanol	40%	Sodium thoisulphate (5% w/v)	10 ml
Nanopure water	50%	Sodium acetate	17 g
		Nanopure water	Added to make up final volume.

Developing solution (500ml)		**Sliver solution**	
Sodium carbonate	12.5 g	Silver nitrate	2.5%
Formaldehyde	200 µl	**Stopping solution**	
Nanopure water	Added to make up final volume.	EDTA	50 mM

Table 2.8: Western blotting solutions

Tris Buffered Saline with Tween (1000.5 mL)		**Western immuno-blot transfer buffer (1000 mL)**	
Tris	9.68 g	Tris	5.82 g
NaCl	32 g	SDS	0.375 g
KCl	0.2 g	Glycine	2.93 g
Tween 20	0.5 mL	Methanol	200 mL
Nanopure water	Added to make up final volume.	Nanopure water	Added to make up final volume.
pH	7.4	pH	9.2

Membrane stripping buffer (100ml)	
SDS	2 g
0.5M tris-HCl (pH 6.8)	12.5 mL
β-mercaptoethanol	0.8 mL
Nanopure water	Added to make up final volume.

Table 2.9: Solutions used in platelet preparation

Tyrodes Buffer		**Citrate**	
NaCl	134 mM	Trisodium citrate $2H_2O$	15.5 mM
KCl	3 mM	**Acid Citrate Dextrose**	
Na_2HPO_4 $12H_2O$	341 µM	Trisodium citrate $2H_2O$	85 mM
$NaHCO_3$	12 mM	Glucose	111 mM
HEPES	20 mM	Citric acid (anhydrate)	78 mM
$MgCl_2$ $6H_2O$	467 µM		
pH	7.3		

Table: 2.10: Solutions used in flow cytometry assays

HEPES Buffer	
NaCl	115 mM
$CaCl_2$	1.2 mM
$MgCl_2$	1.2 mM
K_2HPO_4	2.4 mM
HEPES	20 mM

Table: 2.11: Solutions used in Co-precipitation assays

Phosphate Buffer Saline (PBS) (pH-7.4)		**NP40 Lysis Buffer**	
NaCl	137 mM	NP40	1%
KCl	2.7 mM	NaCl	150 mM
Na_2HPO_4	10 mM	Tris-HCl (pH-8)	50 mM
KH_2PO_4	2 mM		

Table 2.12: Antibodies used in co-immuno precipitation assays

Assay	**Antibody**	**Host**	**Isotype**	**Source**
CD36 co-immuno precipitation	Anti CD36 antibody – FA6-152 (M)	Mouse	IgG	abcam
FcϒRIIa co-immuno precipitation	Anti CD32 antibody – IV.3 (M)	Mouse	IgG	Stem Cell Technology

Table 2.13: Western blotting antibodies/ proteins

Western blot		Primary Antibody	Host	Source	Working Dilution	Iso-type
S. aureus Lipoteichoic acid		*S. aureus* LTA (M)	Humanized	Biosynexus	1:500	IgG
S. aureus Lipoprotein		Biotin Tagged	N/A	Current study	N/A	N/A
VASP	Ser 157	p-VASP antibody – Ser157 (P)	Rabbit	Santa Cruz Biotech	1:1000	IgG
	Ser 239	p-VASP antibody – Ser239 (P)	Goat	Santa Cruz Biotech	1:1000	IgG
14-3-3- zeta		14-3-3 ζ Antibody (P)	Rabbit	Santa Cruz Biotech	1:1000	IgG
Tyrosine phosphorylation		4G-10 (M)	Mouse	Merck Millipore	1:4000	IgG
PKC substrate phosphorylation		Phospho-PKC substrate (M)	Rabbit	Cell Signaling Technology	1:1000	IgG
Integrin β3		Integrin β3 antibody – N20 (P)	Goat	Santa Cruz	1:1000	IgG
CD36		Anti CD36 antibody – FA6-152 (M)	Mouse	abcam	1:1000	IgG
Integrin αIIb		Integrin αIIb antibody – H160 (P)	Rabbit	Santa Cruz	1:1000	IgG
CD9		CD9 antibody C-4 (M)	Mouse	Santa Cruz	1:1000	IgG
FcϒRII		Anti CD32 antibody – IV.3 (M)	Mouse	Stem Cell Technology	1:1000	IgG

Table 2.14: Proteins and antibodies used for aggregometry study.

Assay	Antibody/ Protein	Incubation	Host	Isotype	Source
Effect of *S. aureus* Lipoproteins	*S. aureus* membrane protein extract (TX-114)	10 min	N/A	N/A	Current Study
Effect of MALP-2	MALP-2	10 min	N/A	N/A	Enzo Life Sciences
Human CD36 neutralization	Anti CD36 JC63.1 (azide free) (M)	20 min	Mouse	IgA	Cayman
Human TLR-2 neutralization	Anti Human TLR2 (M)	20 min	Mouse	IgG	eBiosciences
Human SR-BI neutralization	Anti SR-BI antibody (M)	20 min	Rabbit	IgG	abcam

Table 2.15: Antibodies and proteins used in flow cytometry.

Assay	Antibody/ Protein	Labeled	Isotype	Source
S. aureus Lipoproteins Binding	*S. aureus* Lipoproteins	FITC	N/A	Current Study
Human platelet αIIbβ3 activation	PAC-1 antibody	FITC	IgMκ	BD biosciences
Human platelet P-selectin exposure (Granule release)	Anti Human CD62 - P	PE-Cy5	$IgG_1κ$	BD biosciences
Human platelet Fibrinogen Binding	Anti Human Fibrinogen	FITC	N/A	Dako
Mouse platelet αIIbβ3 activation	JON/A antibody	PE	IgG_{2b}	emfret analytics
Mouse platelet P-selectin exposure (Granule release)	Anti CD62P	Alexa Fluro 647	$IgG_{1λ}$	BD biosciences

Chapter 3

S. aureus lipoproteins are involved in interaction with human platelets.

3.1. Introduction

S. aureus modulates platelet function via multiple surface and extracellular molecules that can directly or indirectly interact with platelet surface receptors (O'Brien *et al.* 2002; Fitzgerald, Foster, *et al.* 2006; Fitzgerald, Loughman, *et al.* 2006; Waller *et al.* 2013; Shannon & Flock 2004). In order to affect platelet function via surface molecules, *S. aureus* needs to be in close proximity to bind platelets (Siboo *et al.* 2005) to perturb normal platelet signalling (Fitzgerald, Foster, *et al.* 2006). The number of bacterial surface molecules involved in the interaction with platelets signifies the time and ability of bacteria to alter platelet activation and aggregation (Fitzgerald *et al.* 2006).

S. aureus lipoproteins are one of the major surface associated molecules recognized by host immune cells via toll like receptors (Schmaler *et al.* 2009; Tawaratsumida *et al.* 2009). Platelets express toll like receptors *(F Cognasse et al*. 2005) and scavenger receptors like CD36, CD68 and SRB1 specific for modified plasma lipoproteins (Valiyaveettil & Podrez 2009). Platelet function is altered on interaction of synthetic lipoproteins with platelet TLRs (Blair *et al.* 2009; Kälvegren *et al.* 2010; Rivadeneyra *et al.* 2014) or on interaction of modified lipoproteins with scavenger receptors (Valiyaveettil, Kar, Ashraf, Byzova, Febbraio & E. a Podrez 2008; Magwenzi *et al*. 2015). Given that lipoproteins can modulate the function of human platelets, the experiments described in the following chapter aimed to characterise the effect that immune-modulatory *S. aureus* lipoproteins had on them.

3.2. Results

3.2.1. *S. aureus* lipoproteins contribute to platelet aggregation.

Aggregation of platelets is an essential event in clot formation (Nesbitt *et al.* 2009). On activation a platelet undergoes structural changes during which it spreads (Lee *et al.* 2012) and some surface receptors are activated (Rivera *et al.* 2009). After these structural changes, platelets can bind to each other directly (Hartwig & DeSisto 1991) or via plasma proteins (Ikeda *et al.* 1991) to form aggregates. Measurement of platelet aggregation is an important marker in understanding the effect of molecules on platelet function.

Platelet aggregations were carried out with whole *S. aureus* SH1000 cells (Figure 3.1-A,B,C) and cell wall extracts (Figure 3.2-A,B,C) of wild type and *lgt* strains, grown in chelex treated BHI which has low ion concentrations (Horsburgh *et al.* 2001). The depletion of ions from the media up regulates expression of ion transport systems and most of the lipoproteins are part of these system (Sheldon & Heinrichs 2012) thus the amount of lipoproteins expressed in the chelex treated BHI will exceed the amount found in the normal BHI. These conditions reflect those found *in vivo* (Low *et al.* 2003; O'Connell *et al.* 1997). *S. aureus lgt* showed a growth defect when compared with the *S. aureus* wild type (Stoll *et al.* 2005) (Figure 3.3). *S. aureus lgt* lacks the glyceryltransferase enzyme which is involved in the first step of the common biosynthetic pathway for lipoproteins (Sankaran *et al.* 1994), thus it is deficient in all lipoproteins (Stoll *et al.* 2005). The growth phase of the mutant was matched with the wild type by increasing its incubation time. *S. aureus lgt* showed reduced ability to aggregate (Figure 3.1A, C) the platelets and its cell wall extracts showed significantly longer aggregation lag time compared to the

parental strain (Figure 3.2B, C). Lipotechioc acid (LTA) deficient *S. aureus* strains were not considered for aggregation assays, as LTA on surface did not affect platelets but when in suspension did (Waller *et al.* 2013), thus aggregation response to LTA deficient strain would be same as it would be to the wild type *S. aureus* strain.

3.2.2. Extraction of *S. aureus* membrane proteins.

Phase extraction of *S. aureus* membrane proteins by the Triton-X 114 method was carried out (Figure 3.4A). TX-114 is a non ionic detergent which forms a clear micellar solution in water at 4^{0}C but increased temperature results in its separation into two phases, detergent and aqueous (Sánchez-Ferrer *et al.* 1989). When a suspension of hydrophobic and hydrophilic substances is solubilized in TX-114 and then allowed to separate in two phases, the hydrophobic moieties are present in detergent phase whereas the hydrophilic ones in aqueous phase (Sánchez-Ferrer *et al.* 1989). During the extraction of membrane proteins the degraded *S. aureus* membrane is suspended in 4% TX-114 solution which causes the hydrophobic proteins especially lipoproteins to be present in the detergent phase whereas soluble hydrophilic portions remain in aqueous phase (Li *et al.* 2008; Asanuma *et al.* 2011). By using this method membrane proteins, especially lipoproteins were extracted from *S. aureus* wild type, *S. aureus ΔltaS, S. aureus lgt and S. aureus ΔltaS lgt*. (Figure 3.4A). Western blot analysis of extracted membrane proteins from *S. aureus* wild type and *lgt* strains showed the presence of LTA (Lipoteichoic Acid) contamination (Figure 3.4B). *S. aureus* LTA inhibits platelet activation by increasing intracellular cAMP levels (Waller *et al.* 2013). To avoid LTA contamination a *S. aureus ΔltaS lgt* double mutant was constructed (Figure 3.4B).

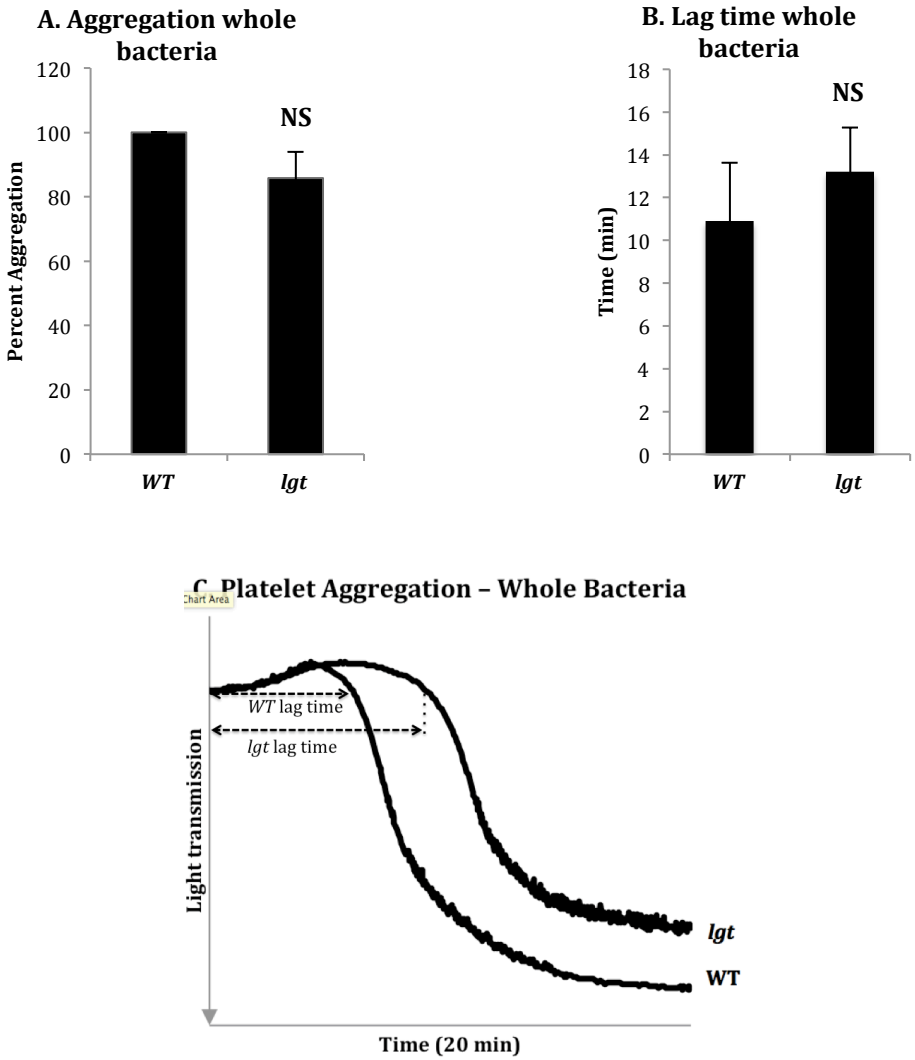

Figure 3.1: Platelet aggregation by* S. aureus *wild type and* lgt *mutants. A. *Data plotted as percent aggregation (wild type treated representing 100%) and represent mean values ± SEM.* ***B.*** *Data plotted as lag time and represents mean value ± SEM.* ***C.*** *Representative aggregation traces for platelets stimulated with* S. aureus *SH1000 wild type (WT) and* lgt *(6 x 10^8 cells/ml). Aggregation was measured for 20 min. (n=3, NS – not significant i.e. P>0.05)*

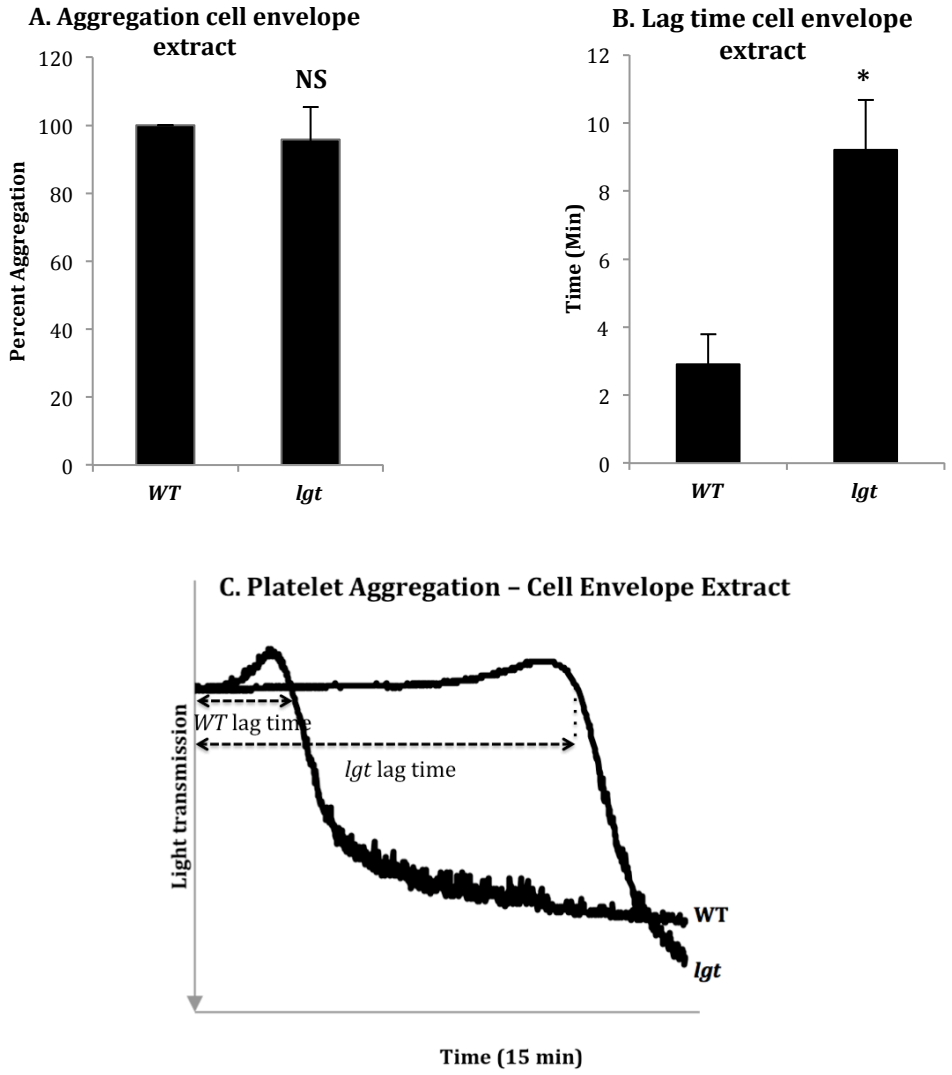

Figure 3.2: Platelet aggregation by* S. aureus *wild type and* lgt *mutants cell envelope extract. A. *Data plotted as percent aggregation (wild type treated representing 100%) and represent mean values ± SEM.* ***B.*** *Data plotted as lag time and represents mean value ± SEM.* ***C.*** *Representative aggregation traces for platelets stimulated with* S. aureus *wild type (WT), and* lgt *cell envelope extracts (500µgml^{-1}). Aggregation was measured for 15 min. (n=3, NS – not significant i.e. P>0.05, *P<0.05 vs.* S. aureus *wild type)*

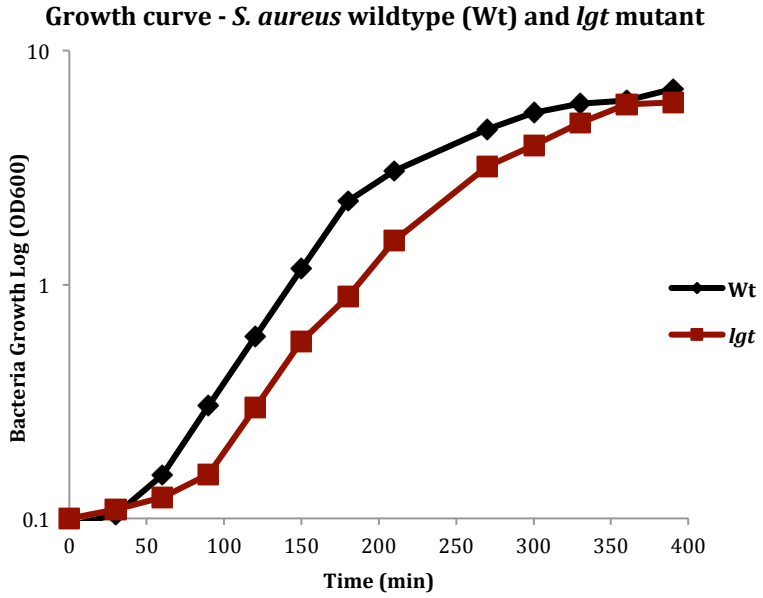

Figure 3.3: S. aureus wild type and lgt mutant growth curve. *S. aureus wild type and* lgt *were inoculated in chelex treated BHI media and growth was checked after every 30 min by recording* OD_{600} *until growth was steady. Graph for growth curve was plotted - log of* OD_{600} *against time (min) for wild type and* lgt *mutant.*

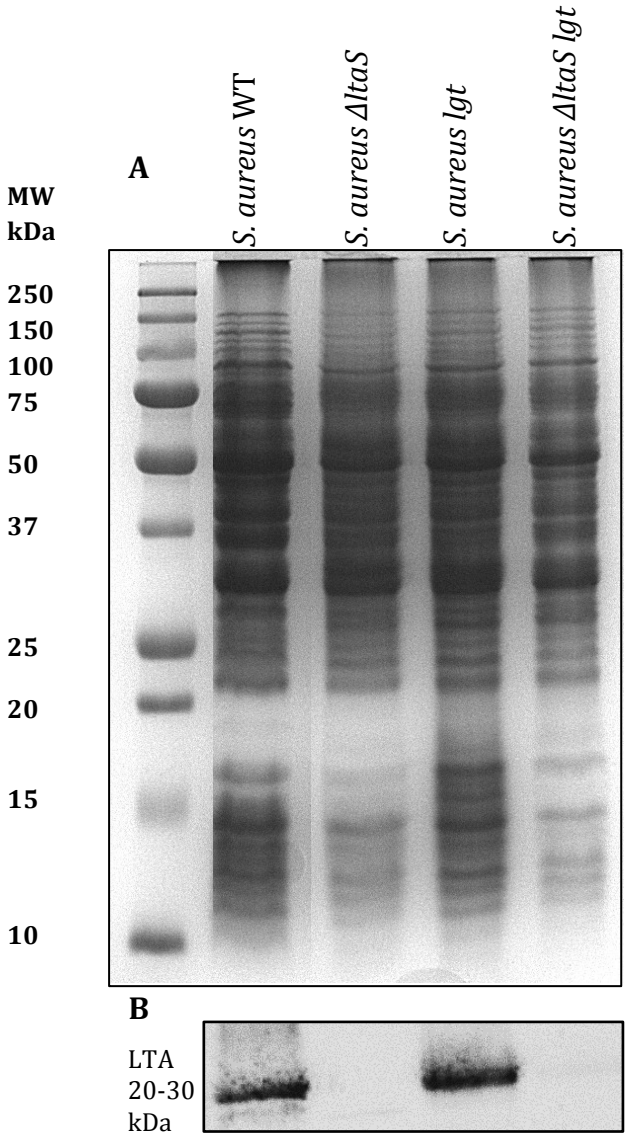

Figure 3.4: LTA free* S. aureus *membrane protein extract. A. *Silver stained SDS PAGE gel of membrane protein extracted from* S. aureus *wild type,* ΔltaS, lgt*, and* ΔltaS lgt *mutant by TX114 phase extraction method.* ***B.*** *Blot for LTA detection in membrane protein extracted from* S. aureus *wild type,* ΔltaS, lgt*, and* ΔltaS lgt *by TX114 phase extraction method.*

3.2.3. *S. aureus* lipoproteins contribute to binding of *S. aureus* to platelets.

To evaluate the contribution of *S. aureus* lipoproteins to binding to platelets, the binding ability of *S. aureus* wild type was compared with *S. aureus lgt* by a binding assay. In comparison to *S. aureus* wild type, *S. aureus lgt* showed decreased ability to bind platelets (Figure 3.5A). The significant reduction in binding of *S. aureus lgt* to platelets suggests that lipoproteins play part in binding of *S. aureus* to human platelets.

3.2.4. Extracted *S. aureus* lipoproteins are capable of binding to platelets.

S. aureus shed lipoproteins during its growth (Stoll *et al.* 2005). To check the ability of these lipoproteins to bind to platelets, membrane proteins extracted from *S. aureus ΔltaS* and *S. aureus ΔltaS lgt* strains were labeled with FITC and their ability to bind with platelets was checked using flow cytometer. Ability of membrane proteins extracted from *S. aureus ΔltaS* was significantly higher as compared to membrane proteins from *S. aureus ΔltaS lgt* (Figure 3.5B), which suggests that lipoproteins directly bind to platelets.

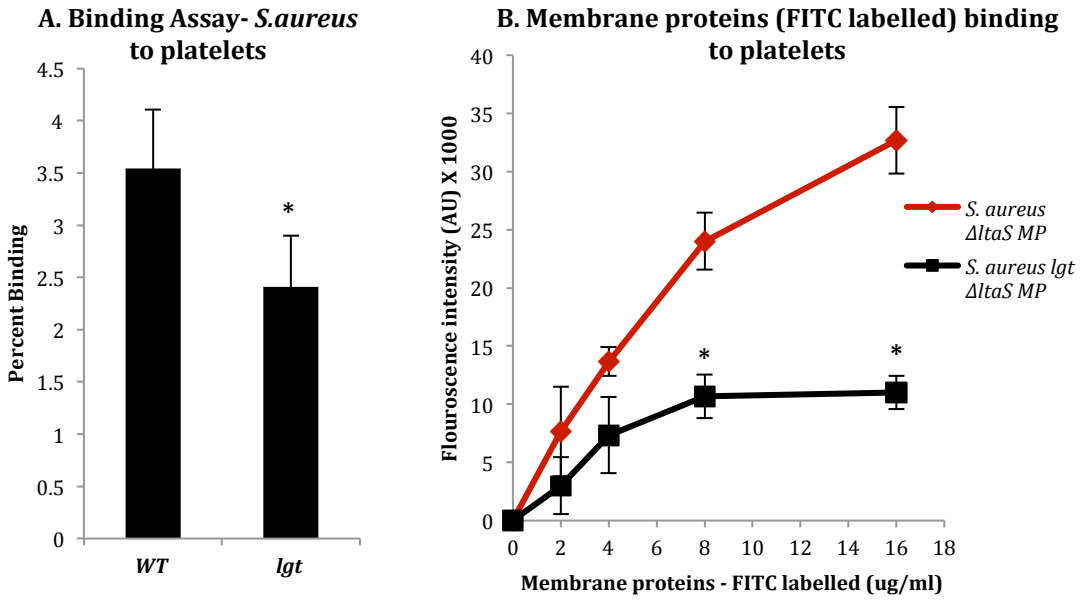

Figure 3.5: **S. aureus *lipoproteins contribute to binding to platelets*. *A.*** *Fixed washed platelets were bound to the plate and bacteria were allowed to adhere. Adhered bacteria were counted and percent binding was calculated. Data plotted as percent binding and represent mean values ± SEM.* ***B.*** *Membrane proteins extracted from* S. aureus ΔltaS and S. aureus ΔltaS lgt *strains were labeled with FITC and added to platelet rich plasma. Mixture was run through flow cytometer. The plot represents median fluorescence intensity recorded at 488nm on gating for platelets and represent mean values ± SEM. (n=3, *P<0.05)*

3.3: Conclusion

Multiple *S. aureus* surface molecules interact with platelets causing their activation and aggregation (Fitzgerald *et al.* 2006). *S. aureus* lipoproteins are one of the major components found on the pathogen surface (Bubeck Wardenburg *et al.* 2006) and are significant in recognition of *S. aureus* by host immune system (Wardenburg *et al*. 2006). Aggregation assays showed that the absence of *S. aureus* lipoproteins increases platelet aggregation lag time (Figure 3.1 – 3.2) which is defined as the time taken to activate the platelets after an agonist (Pulcinelli *et al.* 2004), in this instance bacteria. It is an indication of the time taken by bacteria to activate platelets and depends on two factors; a) difference in affinity of different bacterial surface molecules interacting with platelets, b) density of adhesins on surface of the bacteria (Fitzgerald *et al.* 2006). Thus it reflects the contribution of bacterial surface molecules in the bacteria – platelet interaction. *S. aureus lgt* showed a significant increase in lag time compared to wild type, suggesting the involvement of lipoproteins in the interaction of *S. aureus* with platelets.

Numerous lipoproteins are expressed by *S. aureus* (Sheldon & Heinrichs 2012). TX-114 phase extraction from *S. aureus* showed the presence of numerous proteins (Figure 3.4A). The *S. aureus lgt* strain which lacks the lipoprotein diacylglyceryl transferase (Lgt) enzyme essential for lipidation of pro-lipoproteins is known to express prolipoproteins on its surface (Stoll *et al.* 2005). TX-114 phase extraction isolates the hydrophobic moieties (Sánchez-Ferrer *et al.* 1989). Lipoteichoic acid (LTA) is another surface associated lipid containing a hydrophobic moiety anchored in *S. aureus* membrane (Waller *et al.* 2013), analysis of TX-114 extracts from *S. aureus* SH1000 showed the presence

of LTA whereas the extract from *S. aureus ΔltaS* (Corrigan *et al.* 2011) did not (Figure 3.4B). In order to get the *S. aureus* membrane protein extract that lacked lipoproteins and was not contaminated with LTA, a double mutant *S. aureus ΔltaS lgt* was constructed. The membrane protein extract from *S. aureus ΔltaS lgt* did not show any LTA contamination (Figure 3.4B). So the TX-114 extract from *S. aureus ΔltaS* and *ΔltaS lgt* double mutant were the choice of strains for membrane protein extraction to analyse the effect of *S. aureus* lipoproteins on platelet function.

During coagulation, direct platelet to platelet binding is an essential event (Rivera *et al.* 2009). As in normal coagulation, cell to cell contact is necessary. In the same way, *S. aureus* direct binding to platelets is thought to be relevant to pathogenesis (Fitzgerald, Loughman, *et al.* 2006; Siboo *et al.* 2005). Aggregation assays showed the involvement of *S. aureus* lipoproteins in interaction with platelets. Further experiments showed not only membrane bound *S. aureus* lipoproteins are involved in binding to platelets (Figure 3.5A) but *S. aureus* lipoproteins in the suspension can also bind platelets (Figure 3.5B). These observations suggest that *S. aureus* lipoproteins – membrane bound or into the extracellular milieu, can bind and interact with platelets. Percentage aggregation was not affected in the absence of *S. aureus* lipoproteins (Figure 3.1, 3.2) suggesting that they might not influence platelet function when present on the surface of *S. aureus*. *S. aureus* lipoproteins are released into the extracellular milieu (Stoll *et al.* 2005). Experimental data in this chapter shows that *S. aureus* lipoproteins in solution interact with platelets and when on surface influence the lag time of *S. aureus* mediated aggregation.

Chapter 4

S. aureus lipoproteins inhibit platelet activation.

4.1. Introduction

Different components of *S. aureus* modulate platelet function in different ways. Some are involved in activation (Loughman *et al*. 2005; Fitzgerald, Loughman, *et al*. 2006), some in inhibition (Waller *et al.* 2013) and some only in binding (Sullam *et al*. 1996; L. O'Brien *et al*. 2002). Assays using *S. aureus* lacking lipoproteins showed them to be involved in binding to platelets (Chapter 3). During growth, *S. aureus* immuno modulatory lipoproteins are released into the extracellular milieu (Stoll *et al.* 2005), so the effect of these soluble lipoproteins on platelet activation was examined in this chapter.

Lipoproteins are known to modulate platelet function (Valiyaveettil, Kar, Ashraf, Byzova, Febbraio & E. a Podrez 2008; Fälker *et al*. 2014). Synthetic triacylated lipoproteins Pam3CSK4 are know to activate platelets through TLR2 (Fälker *et al.* 2014; Rivadeneyra *et al.* 2014; Kälvegren *et al.* 2010), but the effect of diacylated lipoproteins is as yet unknown. During their biogenesis in Gram negative bacteria, lipoproteins are triacylated by N-acyltransferase (Lnt) whereas Gram positive bacteria lack an Lnt homolog and produce diacylated lipoproteins (Kovacs-Simon *et al*. 2011; Nakayama *et al.* 2012). *S. aureus*, a Gram positive bacterium, can thus be predicted to produce diacylated lipoproteins (Tawaratsumida *et al.* 2009).

S. aureus lipoproteins stimulate macrophages to release nitric oxide (Kim *et al.* 2015), a vasodilator (Randriamboavonjy *et al.* 2004) and potent inhibitor of platelet activation (Crane *et al.* 2005). *S. aureus* lipoproteins can thus indirectly

inhibit platelet activation, but any direct effects of these lipoproteins on platelets remains unknown.

In order to evaluate their response to *S. aureus* lipoproteins, platelets were preincubated with membrane protein extracted from *S. aureus ΔltaS* and *S. aureus ΔltaS lgt*, then stimulated with thrombin or CRP. Thrombin is a potent activator of platelet activation and coagulation (Kahn *et al.* 1998). On damage to the vessel wall, tissue factors are released which act on zymogen prothrombin to form thrombin (Kuharsky & Fogelson 2001). With the exposed endothelial matrix components playing role in adhesion of platelets (Gardiner *et al.* 2010), a minute amount of thrombin induces their activation. When activated, more platelets are recruited (Offermanns 2006) and more thrombin is formed thus amplifying the response (Sinha *et al.* 1983; Wolberg & Campbell 2008). Along with activation of platelets, thrombin acts on fibrinogen to form fibrin (Kumar *et al.* 1994) which forms mesh like structures with platelets, leading to a stable clot (Weisel 2007). Thrombin is a protease and activates platelets via Protease activated receptors (PAR's), G-protein coupled receptors (Kahn *et al.* 1998; Chen *et al.* 2013). PAR-1 and PAR-4 are known to be responsible for activation of human platelets upon cleavage by thrombin (Kahn *et al.* 1998). PAR-1 can cause activation of platelets at low concentrations of thrombin whereas activation via PAR-4, requires a high concentration of the protease (Kahn *et al.* 1999). An activated PAR leads to activation of Gq (McCoy *et al.* 2012), G12 (Soto *et al.* 2015), and Gi family members (Woulfe *et al.* 2002), which in turn cause activation of PLCβ, PI 3-kinase, and the monomeric G proteins, Rho, Rac, and Rap1, and also increases cytosolic Ca^{2+} concentration (Andersen *et al.* 1999). These sequential events lead to platelet activation.

CRP-XL (collagen related peptide) is an agonist that activates platelets via collagen receptor GPVI (Jung *et al.* 2012). On damage to the vessel wall, endothelial matrix proteins are exposed to the blood stream (Hoylaerts *et al.* 1997; Baruch *et al.* 1991). One of the major proteins present in the matrix is collagen (Hoylaerts *et al.* 1997), which is composed of repeating GPO (Glycine, Proline and Hydroxyproline) triplets spread out throughout the molecule. These GPO triplets are arranged in triple helical structure of collagen in such a manner that are recognized by GPVI (Smethurst *et al.* 2007). CRP-XL consist of 10 repeats of GPO triplets and is made of three pepide chains which cross-link to form collagen like structure that interacts with GPVI, leading to its activation (Smethurst *et al.* 2007).

In the following chapter, the effect of *S. aureus* lipoproteins on platelet aggregation, αIIbβ3 activation, granule release and spreading on fibrinogen, caused after stimulation with thrombin and CRP-XL was examined. The effect on thrombus formation under flow on a collagen surface was checked by pre-incubating whole blood with *S. aureus* lipoproteins. Similarly the effect of synthetic diacylated lipoproteins MALP-2 on human platelets was also studied.

4.2. Results

4.2.1. *S. aureus* lipoproteins inhibit platelet aggregation.

To check the effect of *S. aureus* lipoproteins on platelet aggregation, platelets were incubated with membrane proteins extracted from *S. aureus ΔltaS* and *S. aureus ΔltaS lgt*. *S. aureus* lipoteichoic acid (LTA) is an potent inhibitor of platelet activation (Waller *et al.* 2013), so to avoid LTA contamination the *S. aureus ΔltaS* strain was used. Membrane proteins from *S. aureus ΔltaS* and *S. aureus ΔltaS lgt* did not cause platelet aggregation on their own. But pre-incubation with membrane proteins from *S. aureus ΔltaS* inhibited the aggregation caused by CRP-XL (Figure 4.1A), thrombin (Figure 4.1B) and *S. aureus* SH1000 (Figure 4.1C) in a dose dependent manner, whereas membrane proteins extracted from *S. aureus ΔltaS lgt* had no effect.

On increasing the concentration of CRP-XL, inhibition of platelet aggregation by membrane proteins from *S. aureus ΔltaS* was nullified (Figure 4.1A). Also the *S. aureus ΔltaS* membrane proteins inhibited platelet aggregation caused by a low concentration (0.05 unit) of thrombin. These data suggest *S. aureus* lipoproteins to be weak inhibitors of platelet aggregation.

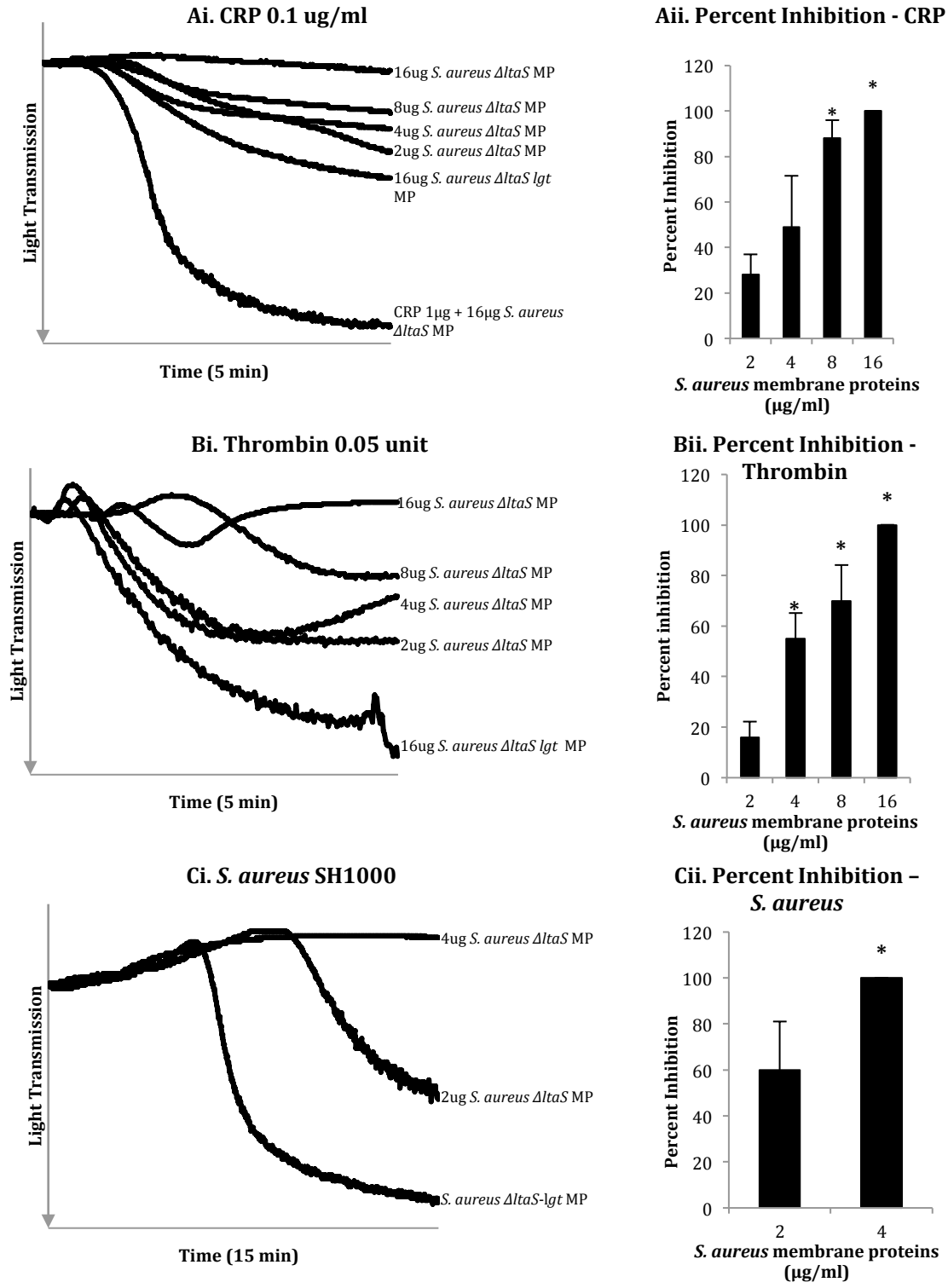

Figure 4.1:* S. aureus *lipoproteins inhibit platelet aggregation. A,B,C: *Representative aggregation traces for platelets preincubated with membrane proteins from* S. aureus *and stimulated with CRP-XL* ***(Ai)****, thrombin* ***(Bi)*** *and* S. aureus *SH1000* ***(Ci)****.* ***Aii,Bii,Cii:*** *Data plotted as percent inhibition (platelets treated* S. aureus *membrane proteins showing complete inhibition represent 100%) and represent mean values ± SEM. (n=3, *P<0.05 vs control – absence of* S. aureus *lipoproteins.)*

4.2.2. *S. aureus* lipoproteins inhibit platelet spreading on fibrinogen.

On activation, platelets undergo shape change and spread (Kita *et al.* 2011). Platelet spreading is essential in coagulation and haemostasis. On spreading, a platelet flattens, increasing the surface area it covers, thus increasing the volume of the clot and sealing the damaged blood vessel (Lee *et al.* 2012). Initial adhesion of platelets to the exposed component of damaged endothelium causes intracellular events leading to integrin activation (Nieswandt *et al.* 2009). When activated, integrin αIIbβ3 can bind to fibrinogen (Payrastre *et al.* 2000). This interaction causes outside in signalling and a rise in intracellular Ca^{2+} that activates kinases responsible for platelet cytoskeleton rearrangement leading to platelet spreading (Lee *et al.* 2012). In this assay, the ability of platelets incubated with *S. aureus* lipoproteins to spread on immobilized fibrinogen was tested.

Spreading of platelets incubated with the *S. aureus ΔltaS* membrane protein extract was significantly inhibited ($P<0.05$ vs control), whereas *S. aureus ΔltaS lgt* membrane proteins had no effect (Figure 4.2). The concentration of *S. aureus ΔltaS* membrane proteins correlated with inhibition. Exposure of platelets to fibrinogen over long time periods did not overcome the inhibition. The number of platelets in the intermediate state of filopodia or lamelopodia was no different compared to control (Figure 4.2B). The majority of platelets bound to fibrinogen existed either in completely spread or non-spread form (Figure 4.2A).

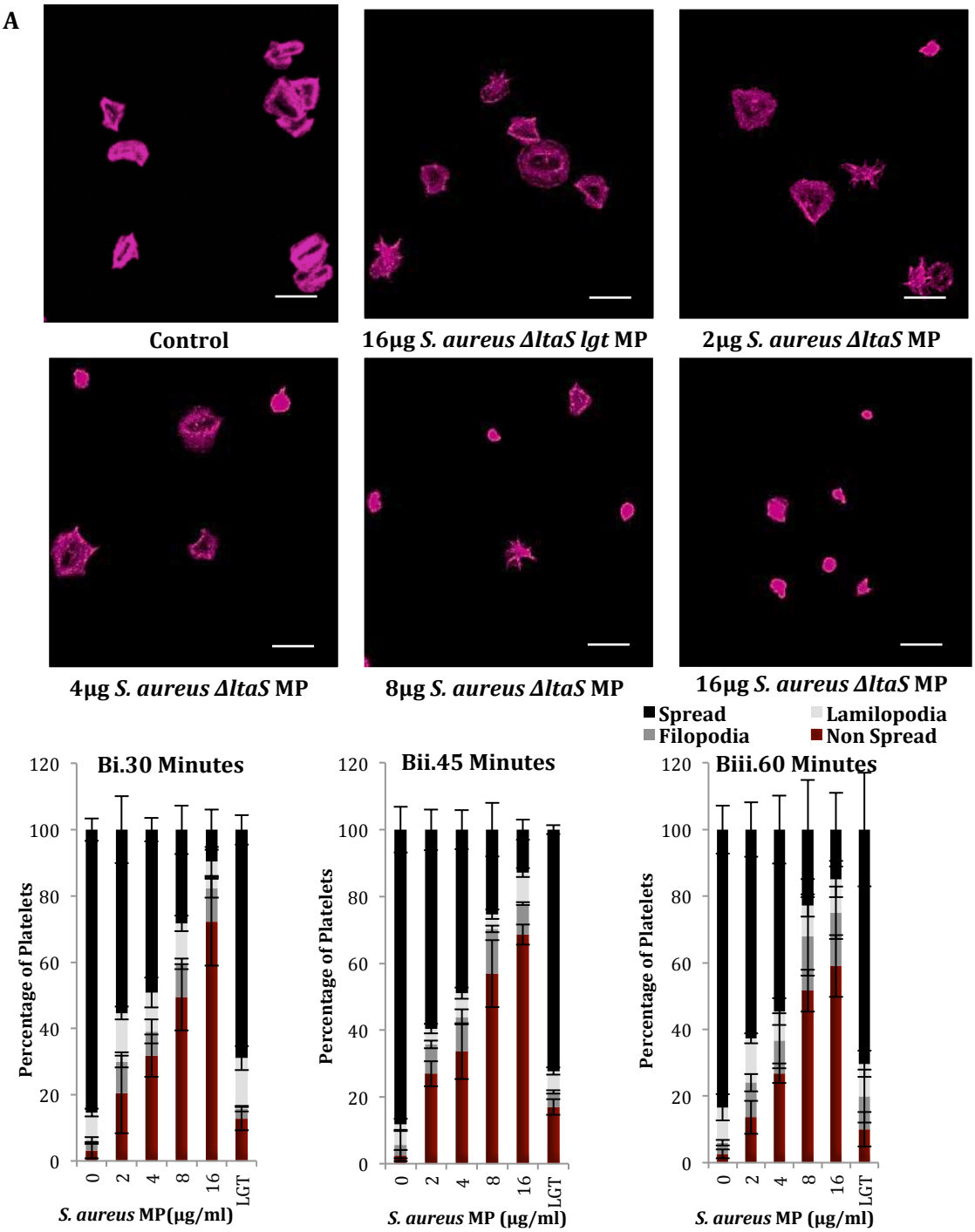

Figure 4.2:* S. aureus *lipoproteins inhibit platelet spreading on fibrinogen. A. *Images taken using fluorescence microscopy of platelet spreading on fibrinogen at different concentration of* S. aureus *membrane proteins. Control represents untreated platelets allowed to spread on fibrinogen. Platelets with labelled with philloidin 647.* ***B.*** *Data plotted as percentage of platelets spread, non-spread, platelets with filopodia and platelets with lamilopodia after allowing platelets incubated with* S. aureus *membrane proteins to spread on fibrinogen for 30 min* ***(Bi)****, 45 min* ***(Bii)*** *and 60 min* ***(Biii)*** *and represent mean values ± SEM. Scale bars – 5µm (n=3).*

4.2.3. *S. aureus* lipoproteins inhibit P-selectin exposure, PAC-1 and fibrinogen binding.

Upon activation platelets undergo changes such as activation of integrin receptors (Nieswandt *et al.* 2009) and release of granules (Flaumenhaft 2003). The major integrin αIIbβ3 (Mehrbod *et al.* 2013) undergoes conformational changes and converts into an activated form (Payrastre *et al.* 2000) which can be recognised by a PAC-1 monoclonal antibody (Taub *et al.* 1989). Binding to coagulation factors and plasma proteins is one of the essential functions of activated integrin αIIbβ3 to facilitate platelet-platelet interaction and stable clot formation (Mehrbod *et al.* 2013). One of the major plasma proteins capable of binding activated αIIbβ3 is fibrinogen (Litvinov *et al.* 2005). Another phenomenon important in platelet activation is the release of granules (Flaumenhaft 2003). α – granules are the major granules in platelets that contains P-selectin molecules on their membrane (Koedam *et al.* 1992). In resting platelets, granules reside in the cytoplasm with P-selectin in the inside of the granule membrane (Furie *et al.* 2001). Upon platelet activation, the granule membrane flips and fuses to the platelet plasma membrane leading to exposure of P-selectin on the surface of platelets (Koedam *et al.* 1992; Furie *et al.* 2001). Thus they are all markers of an activated platelets.

The effect of *S. aureus* lipoproteins on integrin αIIbβ3 activation, fibrinogen binding and P-selectin exposure caused by thrombin (Figure 4.3) and CRP-XL (Figure 4.4) was measured by flow-cytometry. Pre-incubation of platelets with *S. aureus ΔltaS* membrane proteins, inhibited activation of the integrin and binding of fibrinogen in a dose dependent manner. P-selectin exposure, and therefore platelet granule release, was also inhibited on

incubation of platelets with *S. aureus ΔltaS* membrane proteins. Membrane proteins from *S. aureus ΔltaS lgt* had no effect (Figure 4.3 and 4.4), suggesting the involvement of *S. aureus* lipoproteins in the inhibition.

4.2.4. *S. aureus* lipoproteins inhibit intracellular calcium release.

Increased intracellular calcium concentration during platelet activation, is a major step leading to platelet shape change (Paul *et al.* 1999) and various signalling events (Varga-Szabo *et al.* 2009). Stimulus of platelets leads to activation of PLC isoforms (Nonne *et al.* 2005; Lian *et al.* 2005; Banno *et al.* 1998) which hydrolyses phosphoinositide 4-5 bisphosphate to form inositile 1-4-5 triphosphate (IP3) and diacyl glycerol (DAG) (Baldassare *et al.* 1989). IP3 induces release of calcium from intracellular compartments (Baldassare *et al.* 1989; Varga-Szabo *et al.* 2009) and DAG is involved in its entry from extracellular space by activating protein kinases that activate the ion channels (Varga-Szabo *et al.* 2008).

The effect of *S. aureus* lipoproteins on the increased intracellular calcium levels was measured by pre-incubating platelet rich plasma with membrane proteins extracted *S. aureus ΔltaS* and *S. aureus ΔltaS lgt*. Membrane proteins from *S. aureus ΔltaS* inhibited release of calcium in concentration dependent manner (Figure 4.5 A, B). The highest concentration (8 µg/ml) of *S. aureus ΔltaS lgt* membrane proteins did not inhibit calcium release (Figure 4.5 A, B) suggesting involvement of *S aureus* lipoproteins in inhibition of increase in intracellular calcium in platelets.

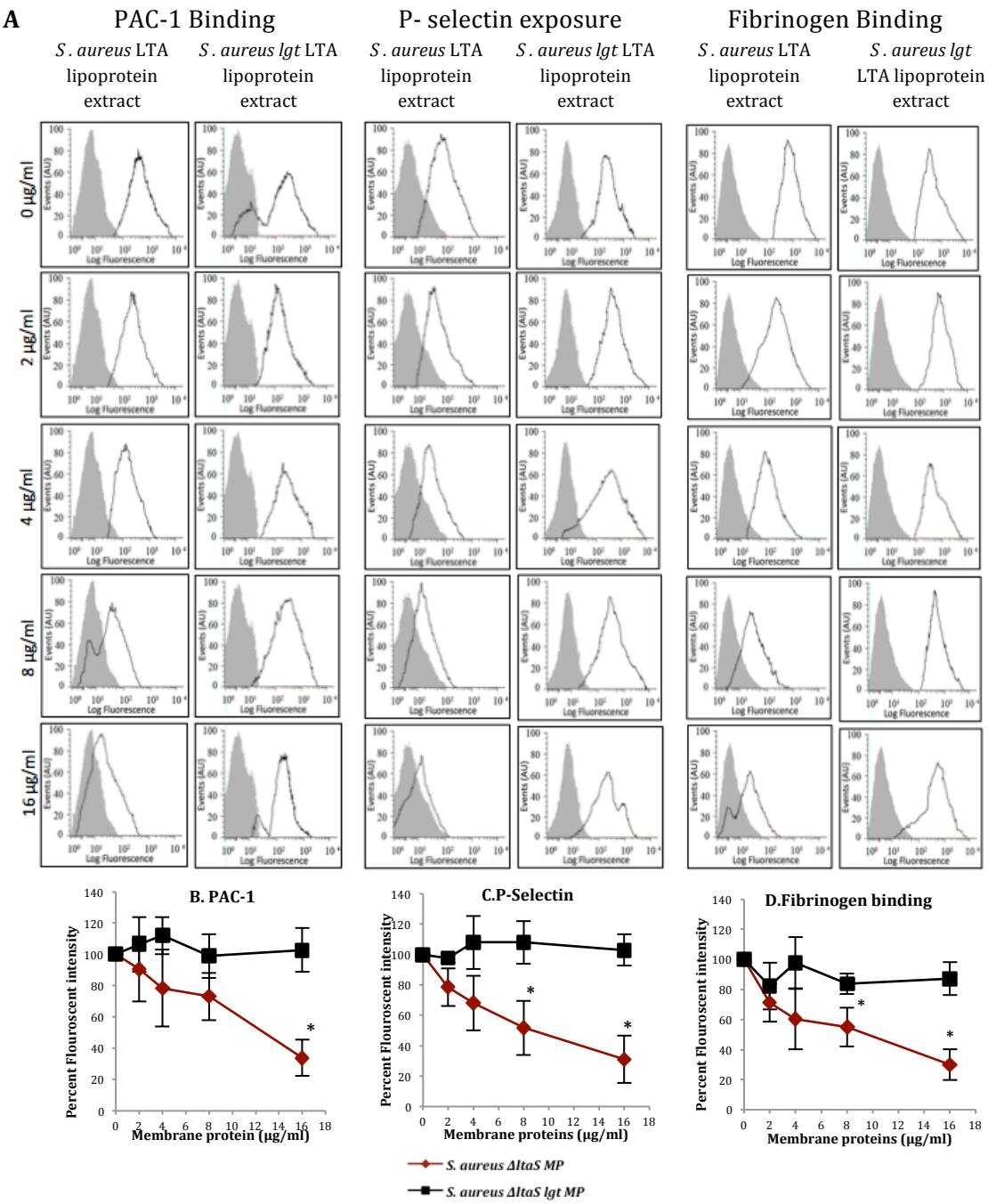

Figure 4.3:* S. aureus *lipoproteins inhibit P- selectin expression, PAC1 and fibrinogen binding of platelets stimulated with thrombin (0.05 unit). (A) *Histograms representing a typical experiment out of three performed with similar results, showing inhibition of PAC1binding, fibrinogen binding and P-Selectin exposure caused by thrombin on preincubation of platelets with* S. aureus *lipoproteins extract in a dose dependent. Grey histogram shows the control non-stimulated platelets. Preincubation of membrane proteins extract from* S. aureus lgt *LTA shows no change in fluorescence shift caused by PAC1binding, fibrinogen binding and P-Selectin exposure on stimulation with thrombin. Data plotted as percentage of flouroscence intensity for binding of PAC-1 antibody* ***(B)****, anti CD62P* ***(C)*** *and anti fibrinogen antibody* ***(D)*** *to platelets. (n=3, *P<0.05)*

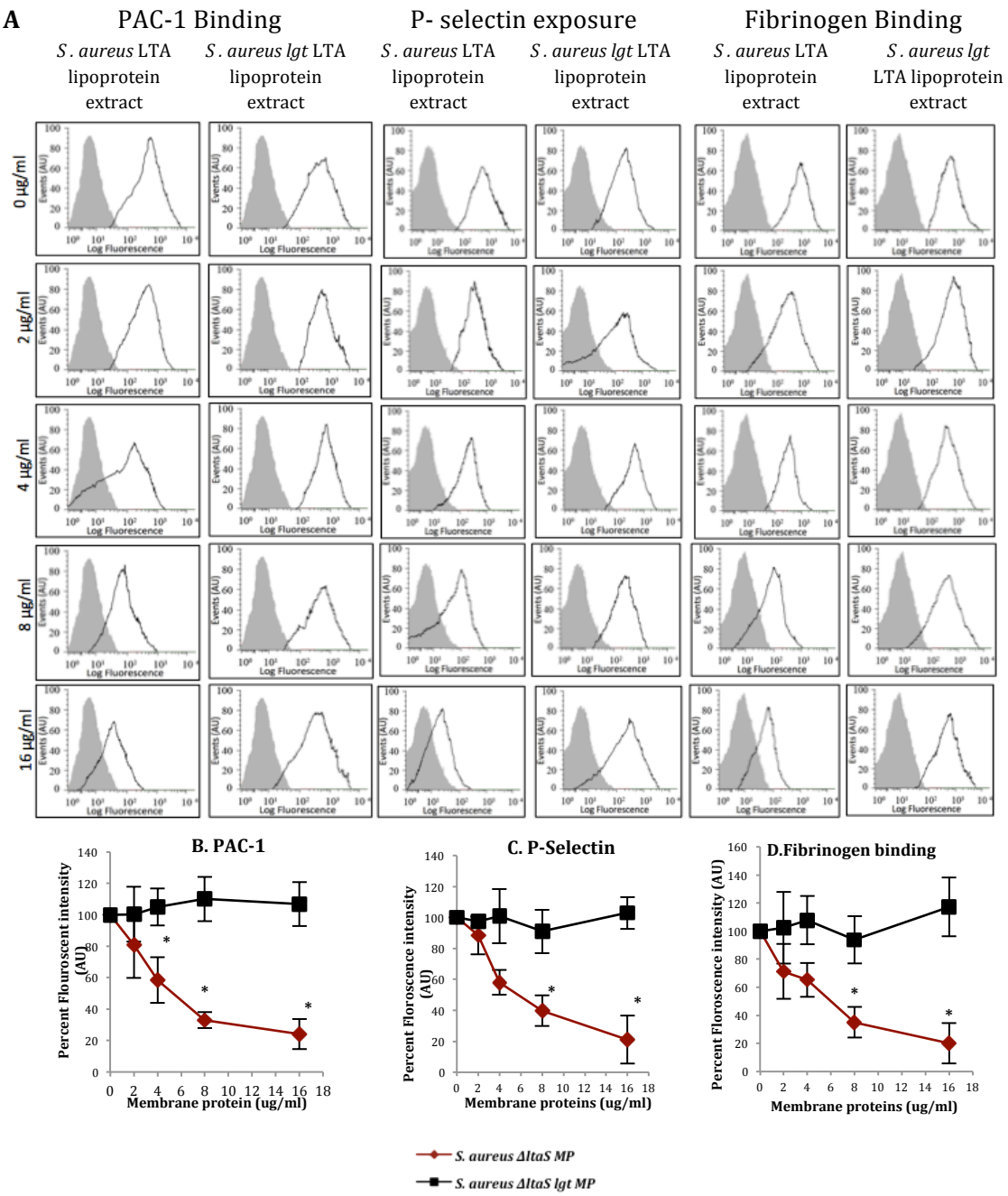

Figure 4.4:* S. aureus *lipoproteins inhibit P- selectin expression, PAC1 and fibrinogen binding to platelets stimulated with CRP-XL. (A) *Histograms representing a typical experiment out of three performed with similar results, showing inhibition of PAC1binding, fibrinogen binding and P-Selectin exposure caused by CRP-XL on preincubation of platelets with* S. aureus *lipoproteins extract in a dose dependent. Grey histogram shows the control non-stimulated platelets. Preincubation of membrane proteins extract from* S. aureus lgt *LTA shows no change in fluorescence shift caused by PAC1binding, fibrinogen binding and P-Selectin exposure on stimulation with CRP-XL. Data plotted as percentage of fluorescence intensity for binding of PAC-1 antibody **(B)**, anti CD62P **(C)** and anti fibrinogen antibody **(D)** to platelets. (n=3, *P<0.05).*

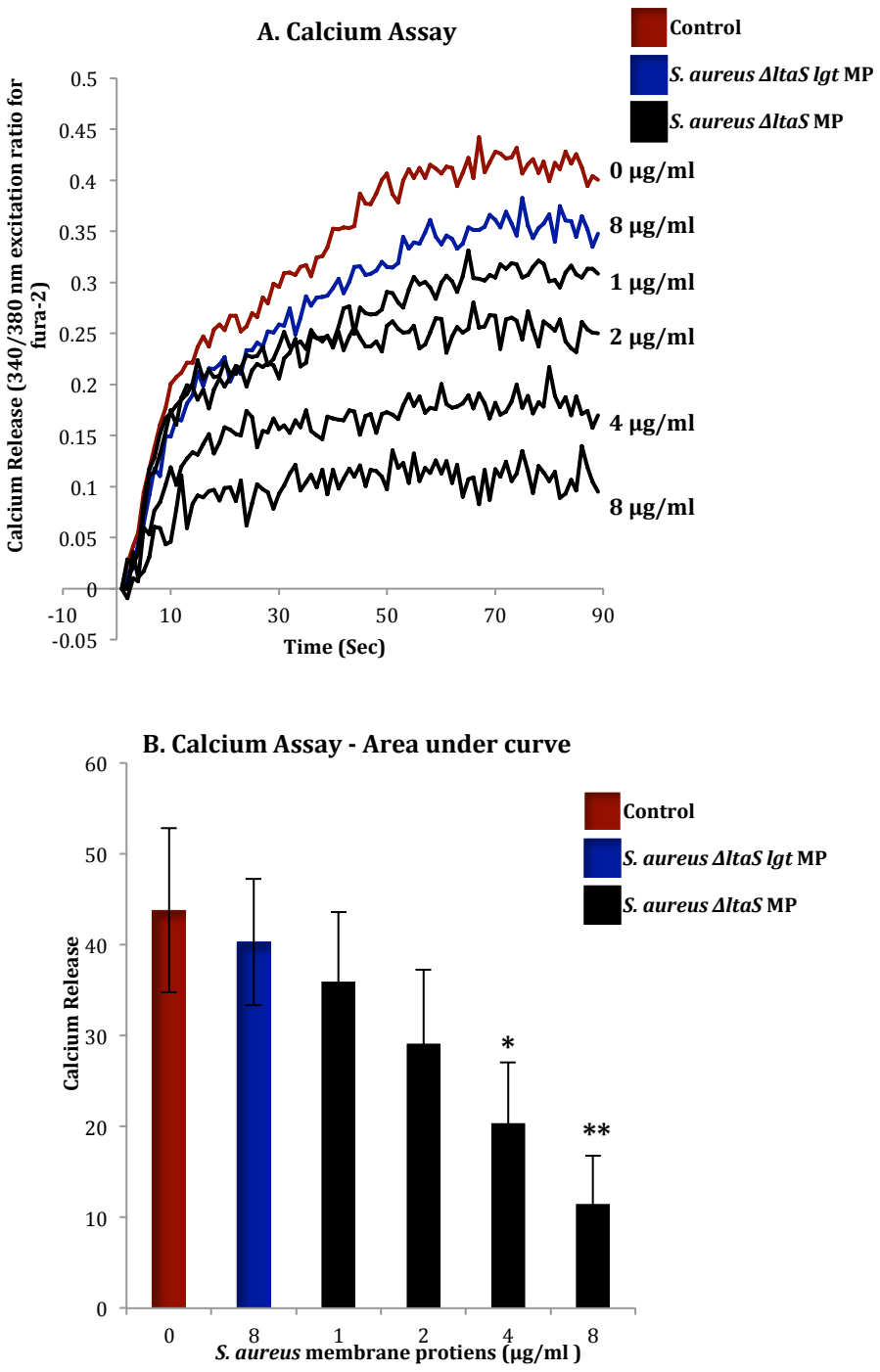

Figure 4.5:* S. aureus *lipoproteins inhibit intracellular calcium release in platelets stimulated with thrombin. *PRP incubated with fura -2 was treated with membrane proteins from* S. aureus ΔltaS lgt *at highest concentration and different concentration of* S. aureus ΔltaS*, then stimulated with 0.1 unit thrombin. Control represents untreated platelets stimulated with thrombin (0.1 unit)* ***(A)*** *Light emission at 340 and 380 nm was measured and 340/380 ratios were plotted against time.* ***(B)*** *Data plotted as area under the curve for graph plotted as 340/380 ratios against time showing the change in intracellular calcium release in platelets. (n=3, *P<0.05, **P<0.01)*

4.2.5. *S. aureus* lipoproteins inhibit thrombus formation under flow.

In-vivo thrombus formation is initiated under constant flow and shear exerted by the vessel wall (Badimon *et al.* 1989; Reininger 2009). In order to depict these *in-vivo* conditions a flow-based assay was used in which a collagen-coated surface acts as the trigger for thrombus formation. Once blood flows over the collagen, initial tethering of platelets to collagen fibers occurs via vWf through platelet glycoproteins GPIb-V-IX and αIIbβ3 (Canobbio *et al.* 2004). Due to this initial tethering, the flow of platelets is restricted allowing collagen receptors GPVI and α2β1 on platelets to bind collagen (Varga-Szabo *et al.* 2008). These interactions lead to intracellular signalling events which cause platelet activation and thus granule secretions that recruits more platelets to the site (Offermanns 2006). In combination with plasma proteins and clotting factors, activated platelets lead to formation of a stable thrombus.

On incubation of blood with membrane proteins extracted from *S. aureus ΔltaS* and *S. aureus ΔltaS lgt*, extracts from *S. aureus ΔltaS* inhibited thrombus formation under flow (Figure 4.6). Extracts from *S. aureus ΔltaS lgt* had no effect on thrombus formation compared to control suggesting that the effect was due to *S. aureus* lipoproteins. The presence of *S. aureus* lipoproteins did not affect initial tethering (Figure 4.6E) of platelets but later stage stable thrombus formation was inhibited (Figure 4.6D).

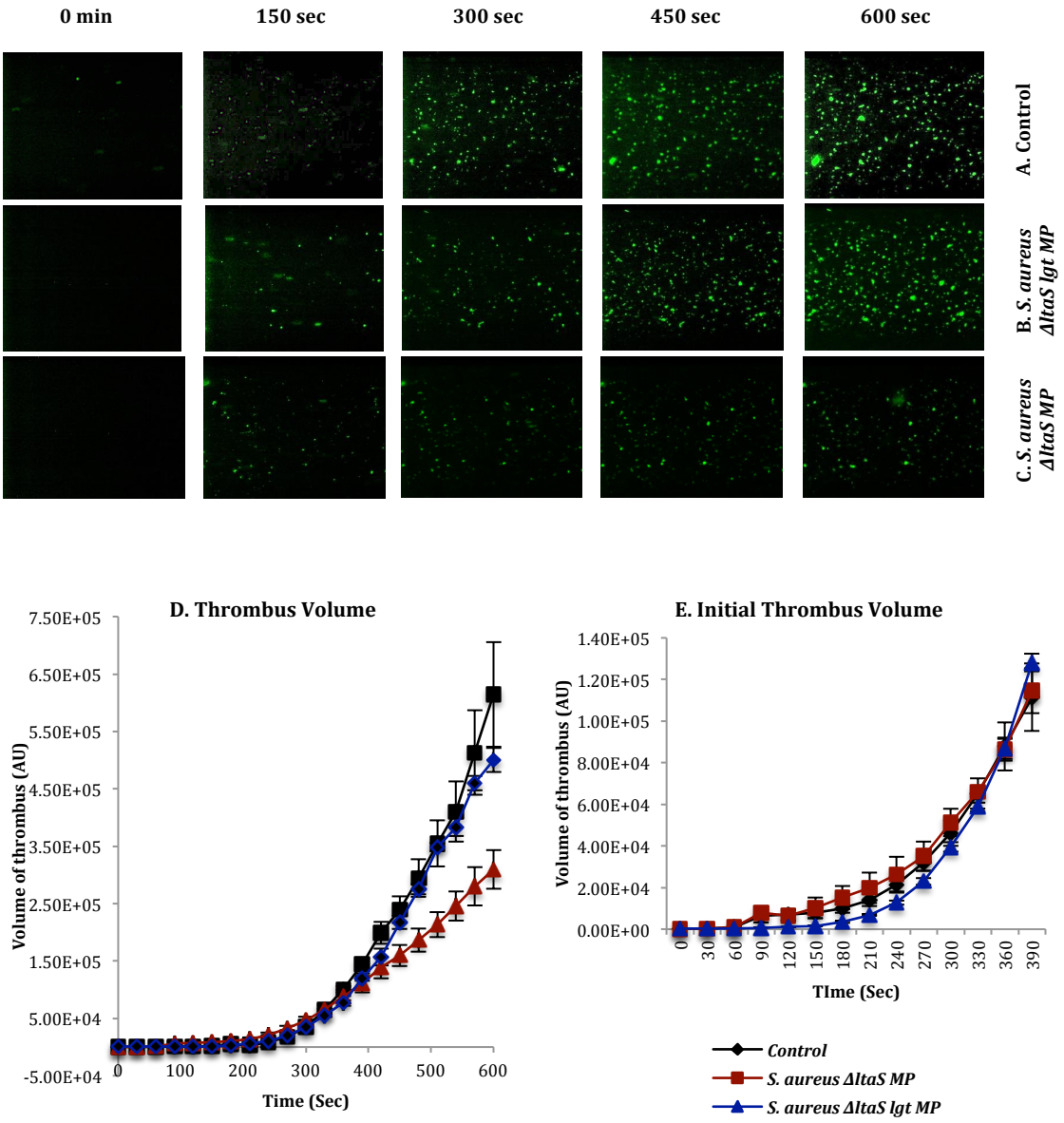

Figure 4.6:* S. aureus *lipoproteins inhibit thrombus formation under flow on collagen-coated surface. *Whole blood was treated with membrane proteins from* S. aureus ΔltaS lgt *and* S. aureus ΔltaS *for 15 min. On treatment, platelets were stained with DIOC6 dye for 5 min and then flowed over the collagen-coated (400 µg/mL) Vena8TMBiochip (Cellex, Dublin) at a shear rate of 20 dynes/cm². Z-stack images of forming thrombi (**A, B, C**) were taken every 30 sec using a Nikon eclipse (TE2000-U) microscope. Volume of thrombus formed (**D,E**) and percent peak fluorescence intensity (**F**) were calculated by analysing thrombus fluorescence intensity using SlidebookTM5 software. (n = 3, *P<0.05)*

4.2.6. Synthetic diacylated lipoprotein MALP-2 inhibits platelet aggregation.

Earlier studies have reported that *S. aureus* lipoproteins are present in both diacylated (Tawaratsumida *et al.* 2009) and triacylated (Asanuma *et al.* 2011) form. But Gram-positive cocci lack the *lnt* gene responsible for triacylation of lipoproteins (Hutchings *et al.* 2009), thus it can be speculated that a mixture of *S. aureus* membrane protein consists predominantly diacylated lipoproteins. In order to see whether this diacylated nature of lipoproteins has an effect on platelets, a diacylated lipoproteins MALP2 was used. MALP2 is a known TLR2/6 agonist, and platelets express both TLR-2 and 6 (Buwitt-Beckmann *et al.* 2005).

MALP2 is a 2 kDa diacylated lipopeptide from *Mycoplasma fermentans* (Borsutzky *et al.* 2005). It is known to activate macrophages via TLR2/6 and cause release of cytokines and chemokines (Galanos *et al.* 2000). It also stimulates macrophages to produce nitric oxide and production of chemo attractants for lymphocytes and leukocytes leading to immune and inflammatory response (Deiters & Mühlradt 1999). MALP2 at low concentration (ng) stimulate production of growth factors accelerating wound healing in mouse and humans (Niebuhr *et al.* 2008). In order to test the effect of MALP2 platelets were incubated with different concentrations of these lipoproteins and stimulated with thrombin or CRP-XL. Platelets pre-incubated with MALP-2 were not able to aggregate on stimulation by CRP-XL (Figure 4.7 Ai,Aii) or thrombin (Figure 4.7 Bi,Bii).

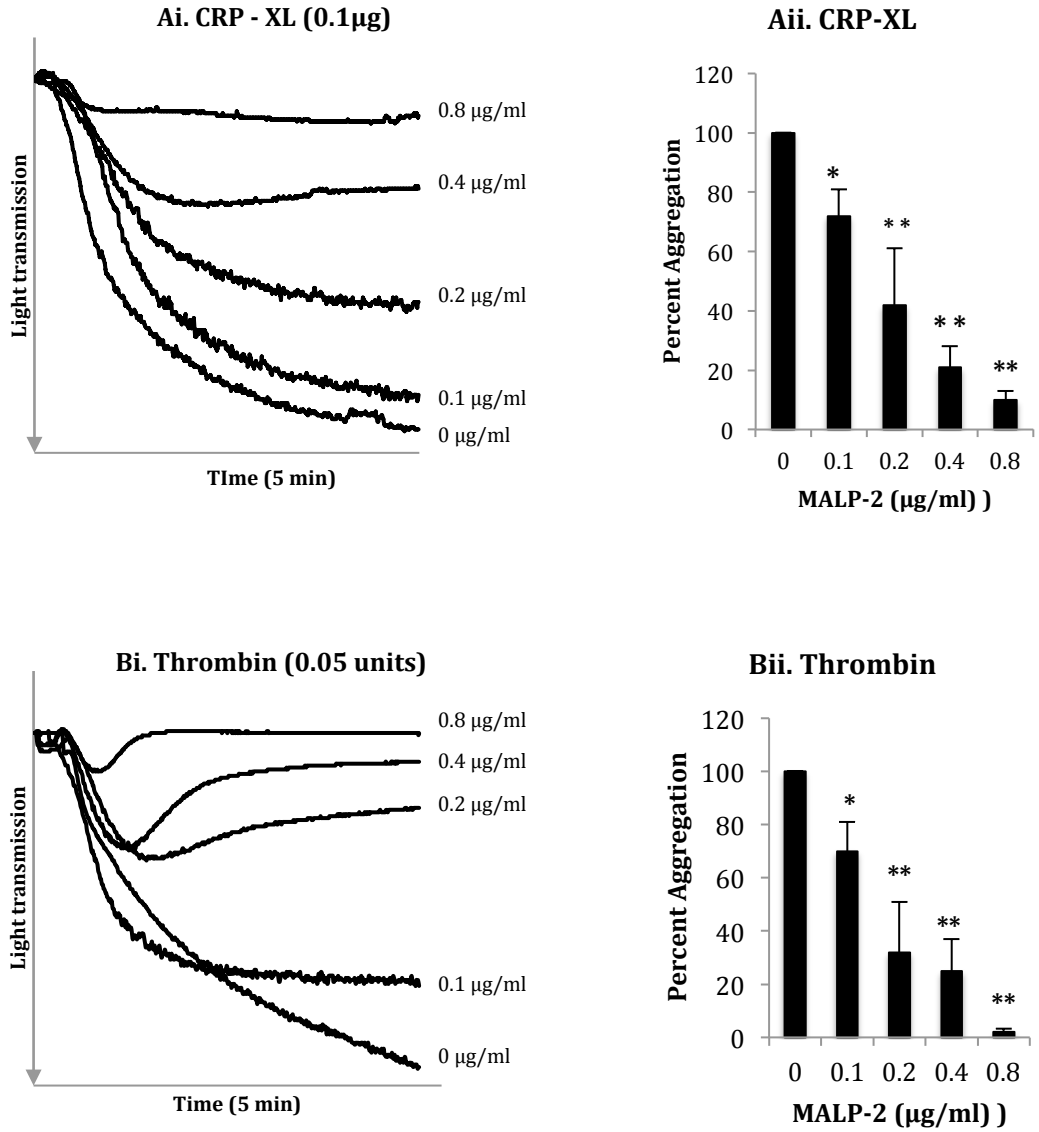

Figure 4.7: MALP-2 inhibits platelet aggregation. A,B: *Representative aggregation traces for platelets preincubated with MALP-2 and stimulated with CRP-XL* ***(Ai)*** *or thrombin* ***(Bi)****.* ***Aii,Bii:*** *Data plotted as percent aggregation – for data representation purpose untreated platelets considered to represent as 100% aggregation and represent mean values ± SEM. (n=3, *P<0.05, **P<0.01)*

4.2.3. Synthetic diacylated lipoprotein MALP-2 inhibits P-selectin exposure, PAC-1 and fibrinogen binding.

The effect of MALP-2 on integrin αIIbβ3 activation, fibrinogen binding and P-selectin exposure caused by CRP-XL (Figure 4.10) was analysed by flow-cytometry. Pre-incubation of platelets with MALP-2 inhibited activation of the integrin and binding of fibrinogen in dose dependent manner (Figure 4.8 B, C). P-selectin exposure thus platelet granule release was also inhibited on incubation of platelets with MALP-2 (Figure 4.8 D).

4.2.7. Mouse platelets are not affected by *S. aureus* lipoproteins.

Mice are the preferred model for haemostasis and thrombosis research (Nieswandt *et al.* 2005) as well as for infections and pathogenesis caused by microbes (Horst *et al.* 2012; Zellweger & Shresta 2014). Most of the insights in function and *in-vivo* changes in platelets are typically done in mice by targeting specific genes (Kahn *et al.* 1998). Despite small variations from human platelets (Ware 2004), mice are considered the most worthwhile model system to study thrombosis.

In order to see whether *S. aureus* lipoproteins have the same effect on mouse platelets as on human, mouse platelet integrin αIIbβ3 activation, and P-selectin exposure caused by thrombin (Figure 4.9 A) and CRP-XL (Figure 4.9 B) was analysed by flow-cytometry. Pre-incubation of mouse platelets with *S. aureus ΔltaS* membrane proteins did not affect activation of mouse integrin αIIbβ3 and platelet granule release.

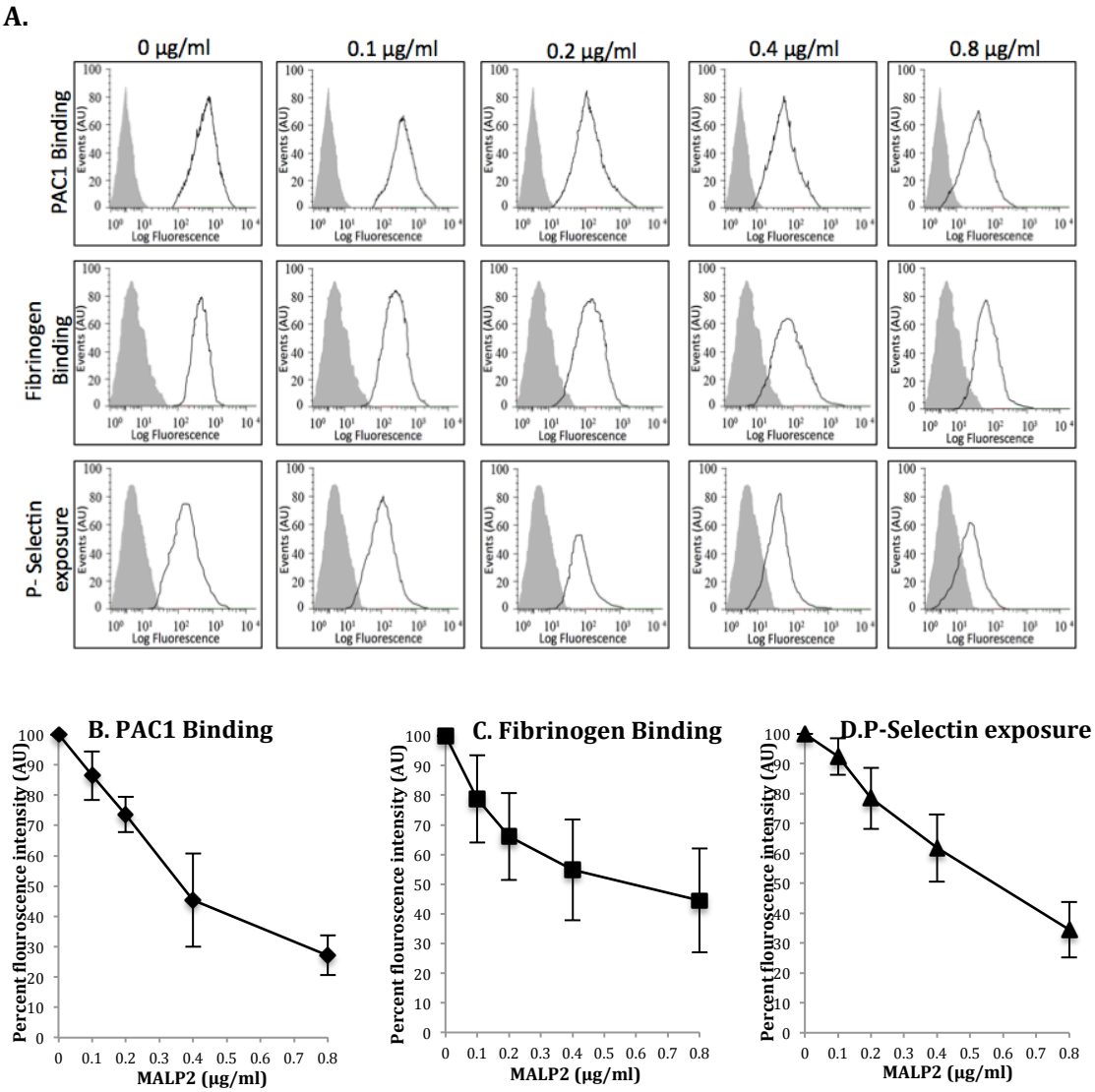

Figure 4.8: MALP-2 inhibits P- selectin expression, PAC1 and fibrinogen binding to platelets stimulated with CRP-XL. *Platelets were incubated with different concentration of MALP-2 and then stimulated with 0.1 µg/ml CRP-XL. Using flow cytometry activation of integrin αIIbβ3 was measured by analysing binding of PAC-1 and anti-fibrinogen antibodies whereas granule secretion was measured by assessing P- Selectin expression using anti CD62P antibody.* ***A.*** *Histograms representing a typical experiment out of three performed with similar results, showing inhibition of PAC1binding, fibrinogen binding and P-Selectin exposure caused by CRP-XL on preincubation of platelets with* S. aureus *lipoproteins extract in a dose dependent. Data plotted as percentage of fluorescence intensity for binding of PAC-1 antibody* ***(B)****, anti CD62P* ***(C)*** *and anti fibrinogen antibody* ***(D)*** *to platelets, represent mean values ± SEM. (n=3, *P<0.05).*

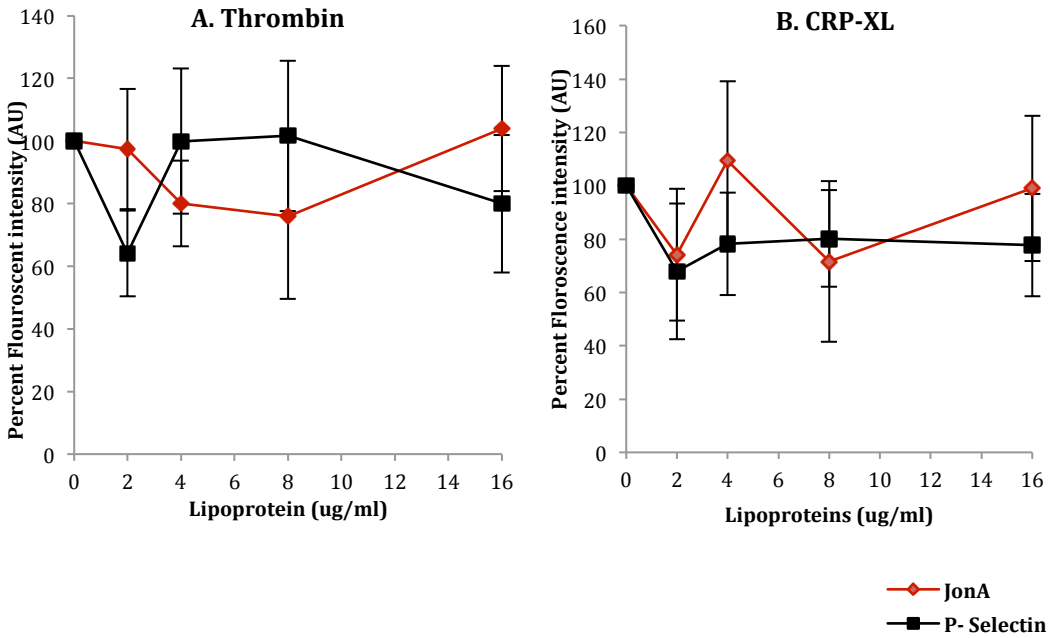

Figure 4.9:* S. aureus *lipoproteins do not inhibit P- selectin expression and JonA binding to mouse platelets stimulated with thrombin and CRP-XL. *Mouse platelets were incubated with different concentration of membrane proteins from* S. aureus ΔltaS *and stimulated with 2 units thrombin and 0.1 µg/ml CRP-XL. Using flow cytometry activation of mouse platelet integrin αIIbβ3 was measured by analysing binding of JonA and granule secretion was measured by assessing P-Selectin expression using anti CD62P antibody. Data plotted as percentage of fluorescence intensity for binding of JonA and anti CD62P antibody on stimulation with thrombin* ***(A)*** *and CRP-XL* ***(B)****, represent mean values ± SEM. (n=4)*

4.3. Conclusion

Activation of platelets leads to direct platelet – platelet interaction and indirect binding through plasma proteins, causing formation of aggregates (Savage *et al.* 1996; Kulkarni *et al.* 2000). Activated integrin αIIbβ3 plays essential role in binding to plasma proteins (Mehrbod *et al.* 2013). *S. aureus* lipoproteins inhibit PAC1 binding (Figure 4.3B, 4.4B) suggesting inhibition of integrin αIIbβ3 activation. On stimulus of platelets by an agonist, initial signalling occurs causing an increase in intracellular calcium and inside-out signalling, leading to conformational change and activation of integrin αIIbβ3 (Mehrbod *et al.* 2013). The initial increase in calcium occurs in the presence of *S. aureus* lipoproteins, but a later sustained increase in calcium is inhibited (Figure 4.5), which would be necessary for complete platelet activation to occur. This inhibition of calcium release and integrin αIIbβ3 activation might be responsible for inhibition of platelet aggregation caused by *S. aureus* lipoproteins.

Release of platelet granules is necessary for recruitment and activation of other platelets at the site of damaged vessel (Flaumenhaft 2003). Among all the granules, α-granules are most prominent and P-selectin expression on the platelet surface is the marker for their release (McGrath *et al.* 2010). *S. aureus* lipoproteins inhibit expression of P-selectin (Figure 4.3C, 4.4C) thus inhibit granule release. Binding of fibrinogen is essential for the platelet clot formation (Kumar *et al.* 1994). The main receptor for fibrinogen on platelets is integrin αIIbβ3 (Mehrbod *et al.* 2013). Binding of fibrinogen to integrin αIIbβ3 induces its clustering which causes outside in signalling leading to complete platelet

activation (Gaul *et al.* 2015). *S. aureus* lipoproteins inhibit fibrinogen binding (Figure 4.3D, 4.4D) thus inhibit integrin αIIbβ3 activation and clustering.

The interaction of fibrinogen and clustering of αIIbβ3 causes outside in signalling leading to platelets spreading (Zhi *et al*. 2015a). Different stages in platelet spreading are the result of different signalling events that occur once integrin αIIbβ3 is activated (Lee *et al.* 2012). In the presence of *S. aureus* lipoproteins, spreading is completely inhibited. Even in the presence of sub-inhibitory concentrations, platelets either exist as completely spread or non-spread. This suggests that on exposure of a platelet to *S. aureus* lipoproteins, outside in signalling is completely blocked. Increased exposure to fibrinogen over time does not affect the inhibition of platelet spreading by *S. aureus* lipoproteins (Figure 4.5). This observation points out that once platelets are exposed to *S. aureus* lipoproteins, they inhibit integrin αIIbβ3 activation and clustering, thus completely inhibiting outside in signalling.

The thrombus formation under flow assay is a close depiction of the *in-vivo* conditions in which thrombi are formed (Roest *et al.* 2011; Harris *et al.* 2015). In the presence of *S. aureus* lipoproteins, stable thrombi were not formed (Figure 4.6). The initial kinetics of thrombus formation, show that the presence of *S. aureus* lipoprotein does not affect the initial binding of platelets to collagen (Figure 4.6E) suggesting that receptors for vWF (GPIb-V-IX) or collagen (GPVI, α2β3) are not affected. The subsequent inhibition of stable thrombus formation suggests that paracrine activation and recruitment of platelets is inhibited which might be due to the fact that in presence of *S. aureus* lipoprotein integrin αIIbβ3 activation and granule secretion is inhibited.

Synthetic diacylated lipoprotein MALP-2 inhibits platelet aggregation, activation of integrin αIIbβ3, binding of fibrinogen and platelet granule secretion. On other cells like macrophages, MALP-2 shows its activity at low concentration (Deiters & Mühlradt 1999) than that used in current study to cause the effect on platelets. This suggests that MALP-2 as like *S. aureus* lipoproteins is also be a weak inhibitor of platelet activation (Figure 4.7, 4.8).

Mouse platelet activation was not inhibited by *S. aureus* lipoproteins. Even though mice are considered as the closest model to humans for platelet studies, there are significant structural and morphological differences between human and mouse platelets (Schmitt *et al.* 2001). Mouse platelets compared to human platelets are smaller in size, differ in granule content (Schmitt *et al.* 2001) and have differences in some surface receptors like PAR's (Sambrano *et al.* 2001). Mouse platelets also lack FcγRIIA, an receptor for IgG present on human platelets (Jirouskova *et al.* 2007). Studies have reported that mouse platelets respond differently compared to human platelets for some molecules binding and affecting αIIbβ3 (Basani *et al.* 2008). The structural difference in mouse and human platelets might be the reason that mouse platelets do not respond to *S. aureus* lipoproteins as human platelets (Figure 4.9).

Overall *S. aureus* lipoproteins are weak inhibitors of platelet activation. Initial binding of platelets to plasma proteins (collagen and fibrinogen) is not affected in the presence of *S. aureus* lipoproteins but later platelet activation and thrombus formation is inhibited. Flow cytometry and spreading data strongly suggests that the activation of major platelet integrin αIIbβ3 is inhibited in the presence of *S. aureus* lipoproteins. Further inhibition of thrombus formation and

P-selectin expression suggests that granule release and recruitment of additional platelets for clot formation is also inhibited in the presence of *S. aureus* lipoproteins. With these assays, it was established that *S. aureus* lipoproteins inhibit platelet activation.

Chapter 5

Inhibition of platelet activation by *S. aureus* lipoproteins is mediated via CD36.

5.1: Introduction

S. aureus lipoproteins are one of the major molecule recognised by the host immune system (Li *et al.* 2008) and a lack of lipoproteins on the *S. aureus* surface reduces the ability of the immune system to clear *S. aureus* infection (Bubeck Wardenburg *et al.* 2006). *S. aureus* lipoproteins are known to interact with TLR2 (Li *et al.* 2008). Localization of *S. aureus* lipoproteins on TRL2 induces signalling via MyD88 in murine macrophages (Schmaler *et al.* 2009) leading to nitric oxide synthesis (Kim *et al.* 2015), interleukin production (Kang *et al.* 2015; Müller *et al.* 2010) and an inflammatory response (Schmaler *et al.* 2009). In human corneal epithelial cells, *S. aureus* lipoproteins cause an innate immune response on binding to TRL2 via NF-κB, JNK, and p38 signalling (Li *et al.* 2008). *S. aureus* lipoproteins also cause activation of caspase 1 and induce IL-1β secretion, promoting haemolysis (Kim *et al.* 2011). Overall, *S. aureus* lipoproteins are capable of binding to host cells, where they induce signalling, causing a physiological response.

Platelet function is affected on interaction with lipoproteins (Rex *et al.* 2009; Ghosh *et al.* 2011). Synthetic triacylated lipoprotein Pam3CSK4 causes platelet activation via TRL2 (Rex *et al.* 2009; Rivadeneyra *et al.* 2014). Stimulation of platelets by Pam3CSK4 causes P2X1-dependent Ca^{2+} mobilisation (Kälvegren *et al.* 2010), activation of src/Syk/LAT/PLCγ2 signalling cascade (Fälker *et al.* 2014) and NF-κB activation (Rivadeneyra *et al.* 2014). Plasma oxidised low-density lipoproteins cause platelet activation via CD36 (Ghosh *et al.*

2011; Nergiz-Unal *et al.* 2011). Interaction between platelet CD36 and plasma oxidised low-density lipoproteins causes activation of protein tyrosine kinase Syk (Nergiz-Unal *et al.* 2011) and CD36/NOX2 mediated inhibition of the cGMP/protein kinase G signalling cascade in platelets (Magwenzi *et al.* 2015). Whereas plasma oxidised high-density lipoproteins interact with SRB1 leading to inhibition of platelet activation and aggregation (Valiyaveettil *et al.* 2008).

Lipoproteins play a significant part in recognition of *S. aureus* by the immune system (Stoll *et al.* 2005) and platelets express the surface receptors for other types of lipoprotein which, on interaction also affect platelet function (Magwenzi *et al.* 2015; Valiyaveettil *et al.* 2008). In the following chapter, a platelet receptor involved in the interaction and in the functional effects of *S. aureus* lipoproteins was identified by co-precipitation, co-immuno precipitation, mass-spectrometry and neutralization assays.

5.2: Results

5.2.1: Mass spectrometry analysis shows CD36 as a major surface protein co-precipitating with *S. aureus* lipoproteins.

In order to identify platelet receptors of *S. aureus* lipoproteins, proteins from platelet lysates were co-precipitated with biotin tagged *S. aureus* lipoproteins and visualized by silver stained SDS PAGE gels. A numbers of bands were visible compared to control that represented entire set of proteins from platelet proteome co-precipitating with *S. aureus* lipoproteins (Figure 5.1). Eight different bands not present in the control were chosen for mass spectrometry analysis. Mass spectrometry analysis provided the score and coverage values for the proteins identified. The coverage value is the percentage of the protein

sequence covered by the identified peptides. The score value is the estimate of how well the mass spectrum matches the expected spectrum for the particular identified protein. Thus high score and coverage values give greater confidence for the identified protein. From the mass spectrometry data, proteins present on the platelet surface found from entire eight bands were analyzed and from them CD36 had the highest score with high coverage (Figure 5.2). CD36 was present in the 80 to 90 kDa range (band 3 – Figure 5.2), which was in the expected molecular weight range (88 kDa) as reported for it (Park 2014).

Other platelet surface proteins found in the entire mass spectrometry prediction were CD109 antigen, GPV, GPVI, GPIbα, GPIbβ and integrin β3. CD109 is considered clinically significant due to the presence of antibodies against it, which are associated with complications such as thrombocytopenia and post-transfusion purpura (Hwang *et al.* 2013; Ertel *et al.* 2005). Co – precipitation of integrin receptors may be significant in the functional effect of *S. aureus* lipoproteins on platelets. However the low score and coverage suggests that it might be co-precipitating with some other receptor (like CD36) with higher affinity for binding *S. aureus* lipoproteins.

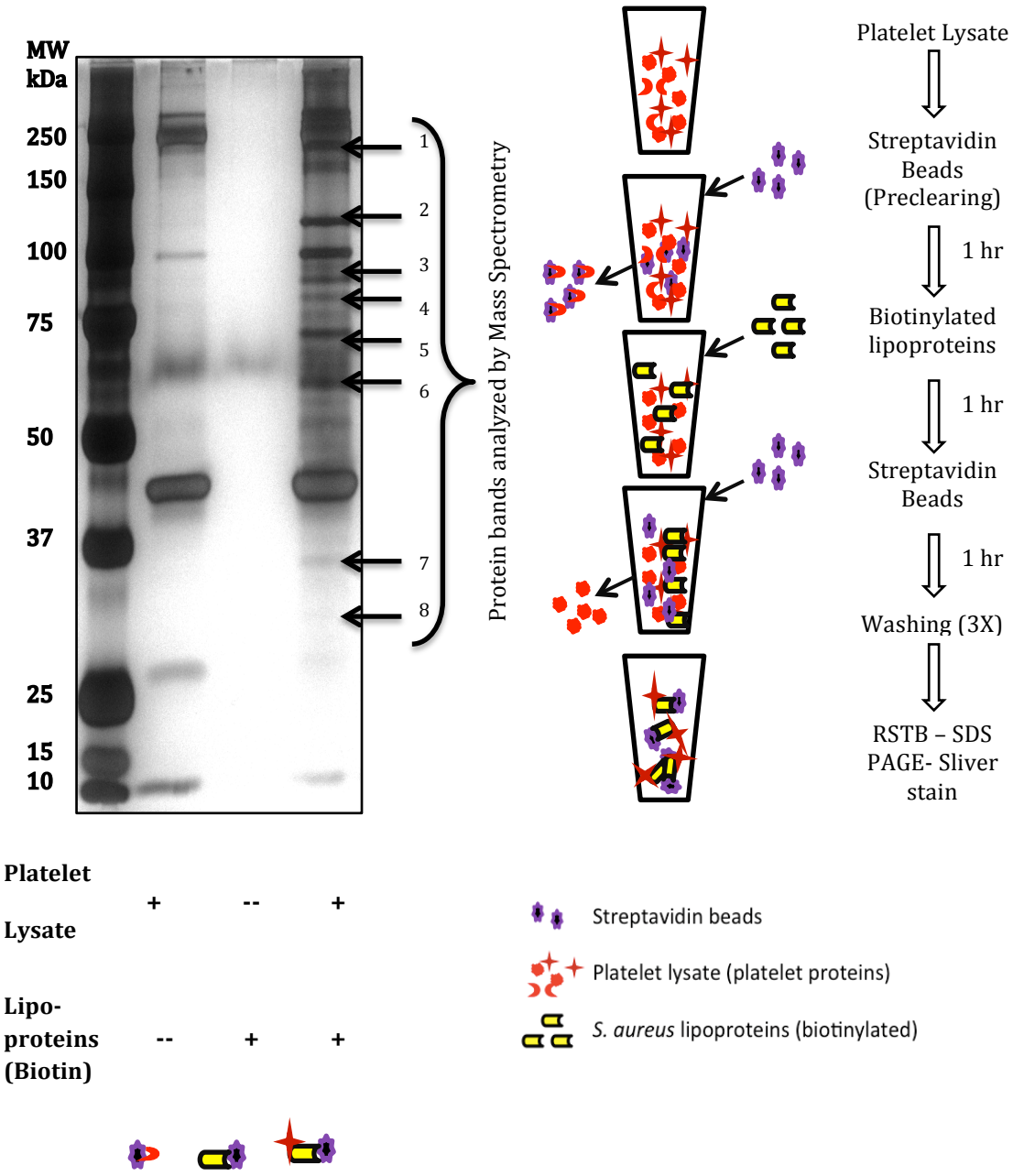

Figure 5.1: Platelet proteins co-precipitated with* S. aureus *lipoproteins. *Platelet lysates treated with biotinylated* S. aureus *lipoproteins. Co-precipitated of S. aureus lipoproteins was done using streptavidin beads. Silver stained SDS PAGE gel showing platelet proteins co-precipitating with* S. aureus *lipoproteins. Eight bands not present in the control were analysed by Mass Spectrometry.*

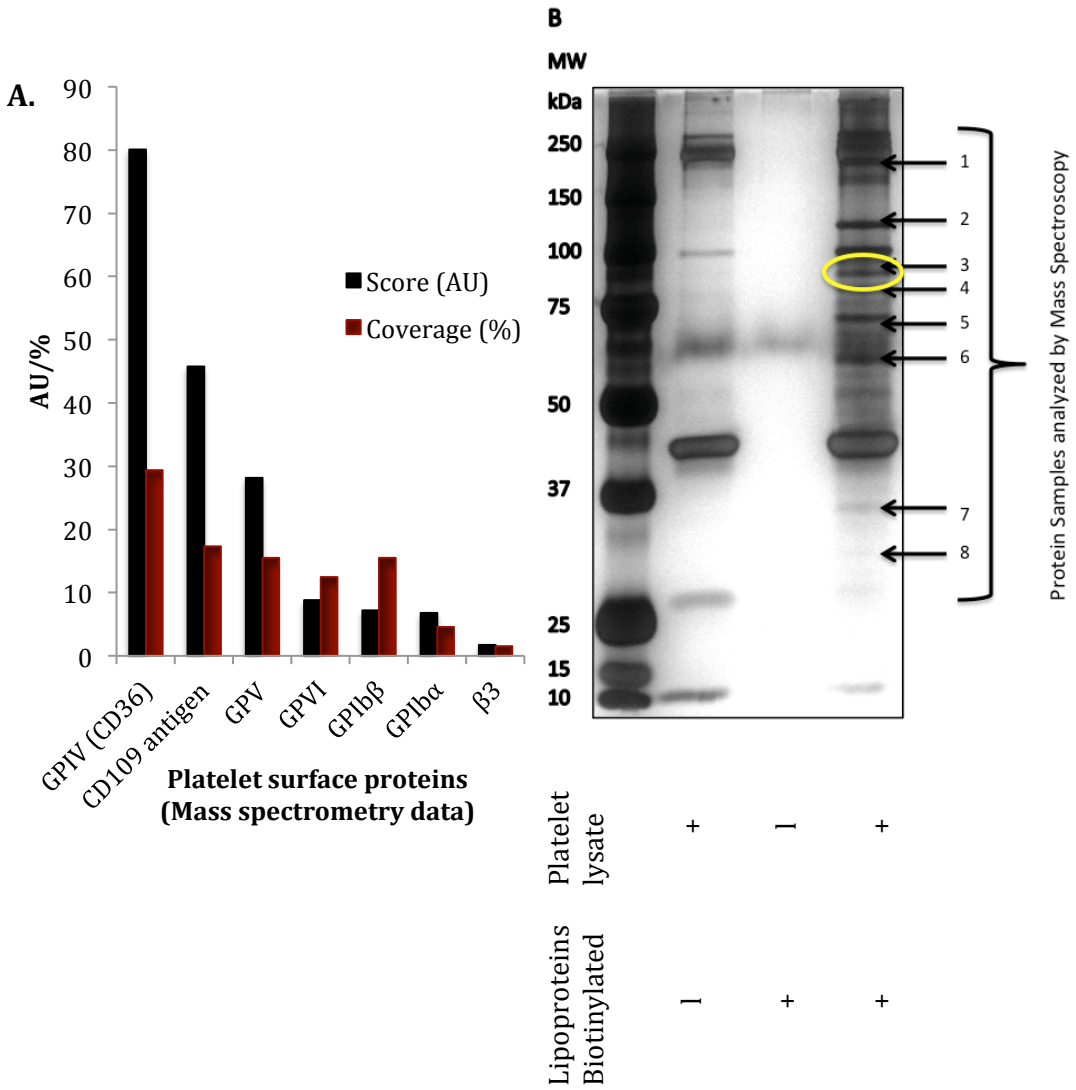

Figure 5.2: Mass Spectrometry analysis for Platelet surface proteins co-precipitated with* S. aureus *lipoproteins. A. *Mass Spectormetry prediction score and protein coverage only for platelet surface proteins obtained from numerous proteins co-precipitated with* S. aureus *lipoproteins. CD36 showed highest score and coverage.* ***B.*** *Silver stained SDS PAGE gel showing platelet proteins co-precipitating with* S. aureus *lipoproteins. Eight bands different form the control analysed by Mass Spectrometry. Band showing presence of CD36 marked in yellow.*

5.2.2: *S. aureus* lipoproteins co-precipitate with platelet CD36.

Mass spectrometry showed that CD36 might be the major platelet surface protein co-precipitating with *S. aureus* lipoproteins (Figure 5.2). To confirm that *S. aureus* lipoproteins bind CD36, a co- immunoprecipitation assay were done. Co-immuno precipitations for CD36 on platelets treated with biotinylated *S. aureus* lipoproteins showed that *S. aureus* lipoproteins bound CD36 (Figure 5.3). Negative controls with only magnetic beads, anti-CD36 antibody and *S. aureus* lipoproteins (Figure 5.3 – band 1 in the western blot) did not show any bands which rules out any non-specific binding and suggests that the *S. aureus* lipoproteins are co-precipitated with platelet CD36. In comparison with the control, nearly all biotinylated *S. aureus* lipoproteins were co-precipitated with platelet CD36 thus confirming its binding ability to *S. aureus* lipoproteins.

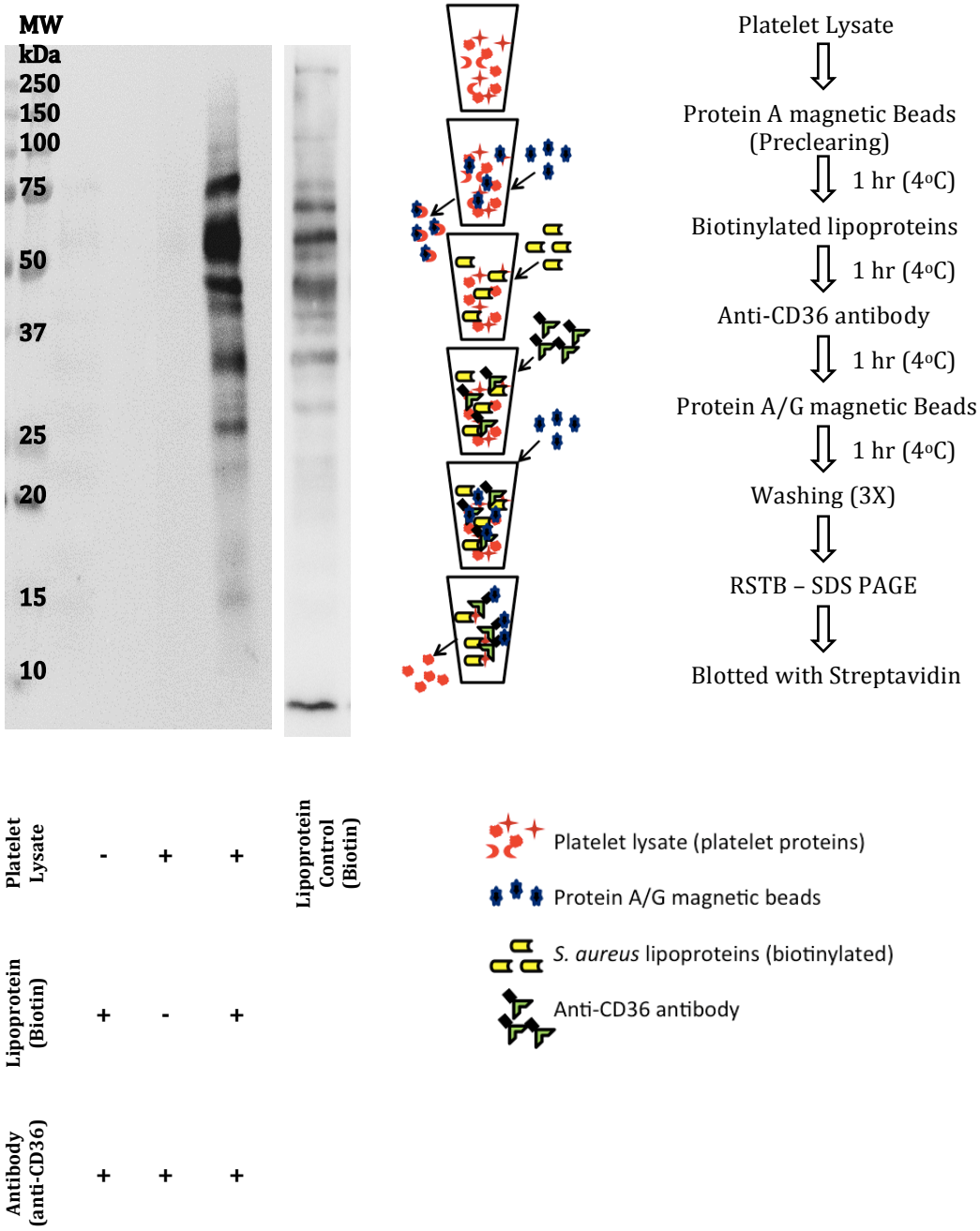

Figure 5.3:* S. aureus *lipoproteins co immuno precipitated with platelet CD36. *Platelet lysate treated with biotinylated* S. aureus *lipoproteins plus anti-CD36 antibody and co-immunoprecipitated with protein A/G magnetic beads. Co-immunoprecipitated proteins were blotted and developed with streptavidin HRP. Western blot showing* S. aureus *lipoproteins co-precipitating with platelet CD36. Lipoprotein control shows streptavidin HRP blotted entire population of biotinylated* S. aureus *lipoproteins.*

5.2.3: Neutralisation of platelet TLR2 or SRB1 does not abrogate inhibitory effect of *S. aureus* lipoproteins on platelet aggregation.

Synthetic lipoprotein Pam3CSK4 interact with TLR2 and cause platelet actvation (Fälker *et al.* 2014), whereas high density oxidised plasma lipoproteins interact with SRB1 (Valiyaveettil *et al.* 2008) and inhibit platelet function. But mass spectrometry data from co-precipitation of platelet proteins with *S. aureus* lipoproteins did not show the presence of TLR2 or SRB1 (Figure 5.2). To confirm that TRL2 and SRB1 are not involved in inhibition of CRP-XL mediated platelet activation caused by *S. aureus* lipoproteins, neutralisation assays were done. Anti TLR2 and anti SRB1 antibodies did not significantly affect CRP-XL mediated platelet aggregation. After treating platelets with monoclonal anti-TRL2 or anti-SRB1 antibodies, and then with *S. aureus ΔltaS* membrane proteins, platelets were stimulated with CRP-XL. Antibodies reactive to TLR2 or SRB1 had no affect on the platelet inhibitory effect of S. aureus lipoproteins (Figure 5.4), suggesting that neither receptor is involved.

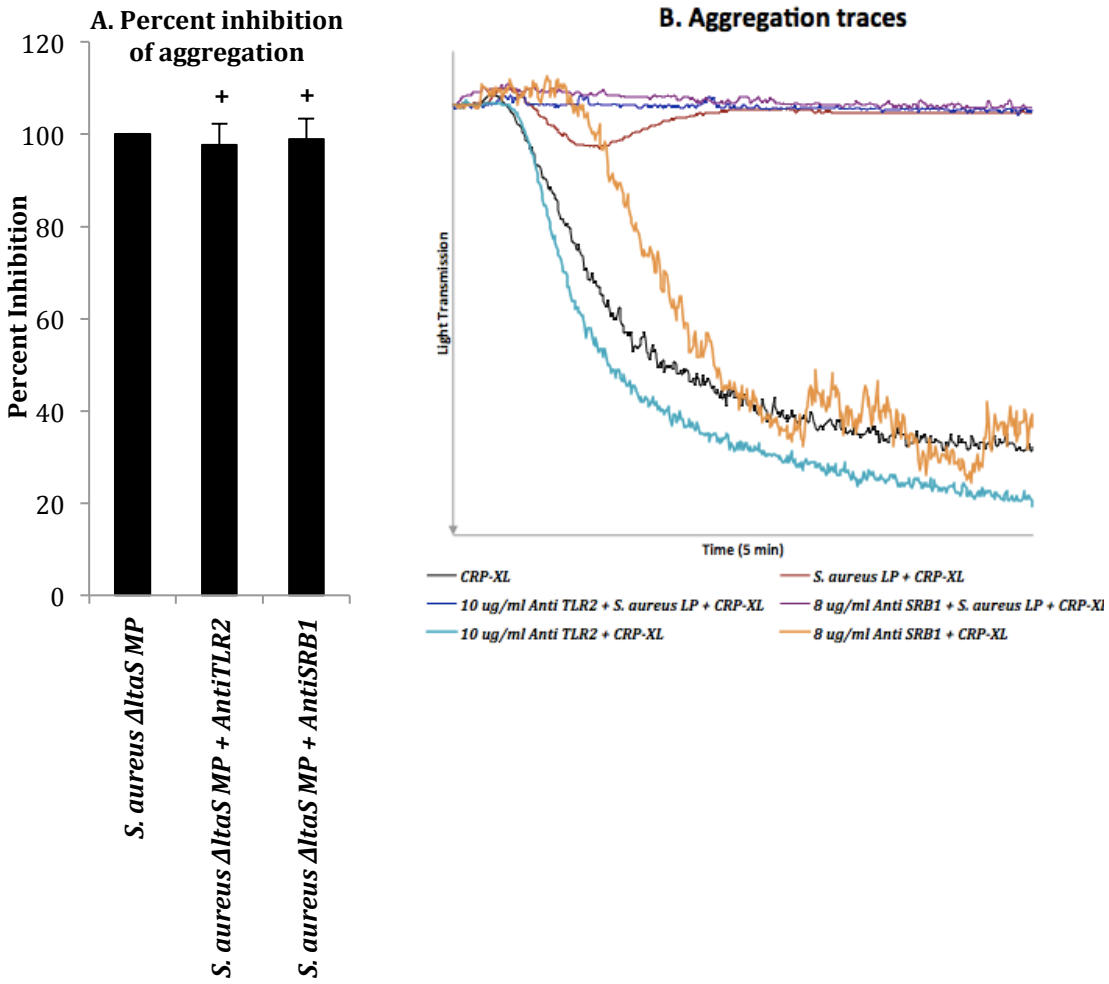

Figure 5.4: Inhibition of platelet aggregation by* S. aureus *lipoproteins is not mediated via TRL2 or SRB1. A. *Data plotted as percentage of inhibition of platelet aggregation caused by* S. aureus ΔltaS *membrane proteins on pre-incubating platelets with anti TLR2 or anti SRBI and then stimulating with CRP-XL. Data represent mean values ± SEM.* ***B.*** *Representative traces of platelet aggregation caused by CRP-XL on its own, on platelets treated with* S. aureus ΔltaS *membrane proteins and on platelets pre-incubated with anti TLR2 or anti SRBI and treated with* S. aureus ΔltaS *membrane proteins. (n=3, ⁺P > 0.05)*

5.2.4: *S. aureus* lipoproteins inhibit platelet aggregation caused by plasma oxidised LDL.

Plasma oxidised LDL is known to cause platelet aggregation via CD36 (Magwenzi *et al.* 2015). On neutralising platelet CD36 with anti-CD36 antibody, platelet aggregation caused by plasma oxidised LDL (10 μg/ml) was inhibited (Figure 5.5A). In the same way, incubating platelets with *S. aureus ΔltaS* membrane proteins, also inhibited platelet aggregation caused by plasma oxidised LDL in a dose dependent manner (Figure 5.5B). There was no effect of treatment with isotype control and *S. aureus ΔltaS lgt* membrane proteins on platelet aggregation caused plasma oxidised LDL (Figure 5.5 A, B).

5.2.5: Neutralisation of platelet CD36 abrogates inhibitory effect of *S. aureus* lipoproteins on platelet aggregation.

On establishing that CD36 is a platelet receptor for *S. aureus* lipoproteins, the next step was to test whether CD36 is responsible for the functional inhibition of platelet activation. On neutralising platelet CD36 using anti-CD36 antibody, inhibition of platelet aggregation caused by *S. aureus* lipoproteins was nullified (Figure 5.6). Platelets aggregated normally in the presence of anti-CD36 antibody. There was no effect on *S. aureus* lipoproteins mediated inhibition of platelet aggregation after treatment with isotype control antibody (Figure 5.6B).

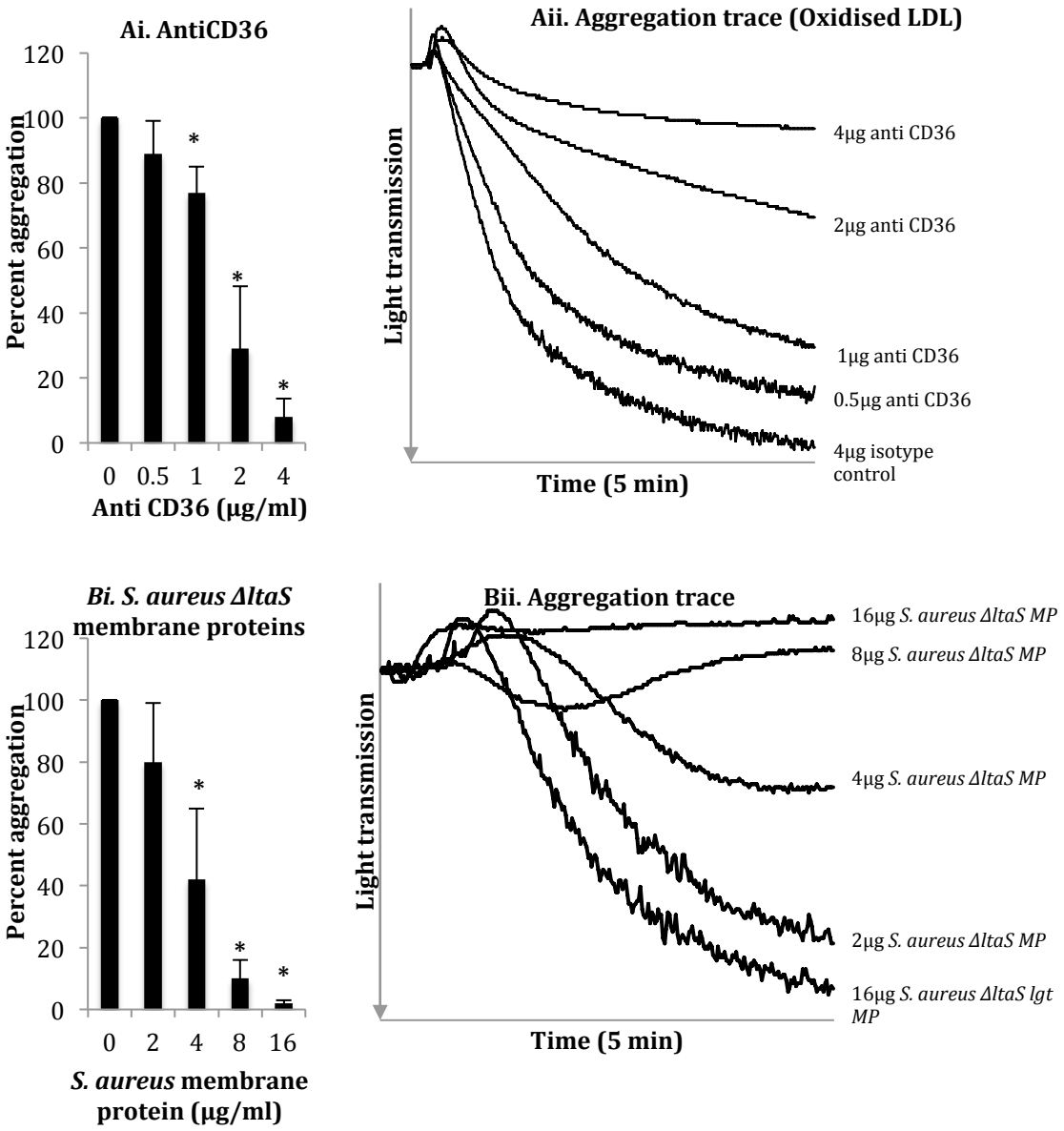

Figure 5.5:* S. aureus *lipoproteins inhibit platelet aggregation caused by oxidized LDL. Ai,Bi. *Data plotted as percent aggregation caused by plasma oxidised LDL (10 µg/ml) on pre-treatment of platelets with anti-CD36* ***(Ai)*** *and* S. aureus *membrane proteins* ***(Bi)****. Data represent mean values ± SEM. (n=3, *P<0.05 vs isotype control)* ***Aii,Bii.*** *Representative aggregation traces for platelets pre-incubated with anti-CD36* ***(Aii)*** *and membrane proteins from* S. aureus ***(Bii)*** *and stimulated with plasma oxidised LDL.*

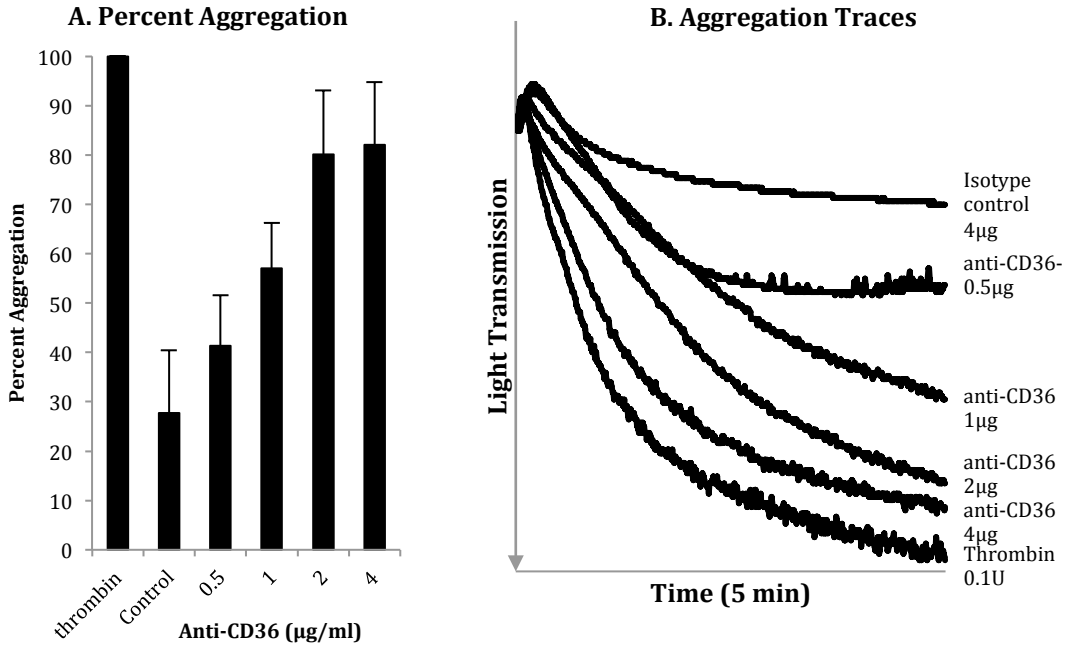

Figure 5.6: Inhibition of platelet aggregation by* S. aureus *lipoproteins is inhibited on neutralisation of CD36. A. *Data plotted as percentage of platelet aggregation on pre-incubating platelets with increasing concentration of anti-CD36, then with* S. aureus ΔltaS *membrane proteins (8 µg/ml). Control shows the data for isotype control antibody (4 µg/ml). Data represent mean values ± SEM.* ***B.*** *Representative platelet aggregation traces caused by thrombin - on its own, on pre-incubating platelets with anti-CD36, then with* S. aureus ΔltaS *membrane proteins. (n=3, *P<0.05 vs isotype control)*

5.2.6: Neutralisation of platelet CD36 abrogates inhibitory effect of *S. aureus* lipoproteins on platelet spreading on fibrinogen.

On neutralising platelet CD36 with anti-CD36 antibody, inhibition of spreading by *S. aureus* lipoproteins was nullified (Figure 5.7 A-B). Platelets treated with *S. aureus* lipoproteins spread normally in presence of anti-CD36 antibody and there was no effect upon treatment with isotype control antibody. As the concentration of anti-CD36 antibody was increased, inhibition of platelet spreading was reduced in a dose dependent manner (Figure 5.7 A).

5.2.7: Neutralisation of platelet CD36 abrogates inhibitory effect of *S. aureus* lipoproteins on platelet P-selectin exposure, PAC-1 and fibrinogen binding.

S aureus lipoproteins inhibit activation of platelet integrin αIIbβ3 and platelet granule release. On neutralising platelet CD36 with anti-CD36 antibody it was seen that inhibition of PAC-1 binding (Figure 5.8A), fibrinogen binding (Figure 5.8B) and P- selectin exposure (Figure 5.8C) caused by *S. aureus* lipoproteins, was nullified. There was no effect of treatment with isotype control antibody. The effect of anti-CD36 occurred in a dose dependent manner. These data suggests that the inhibitory effect of *S. aureus* lipoproteins on integrin αIIbβ3 activation and platelet granule release is mediated via CD36.

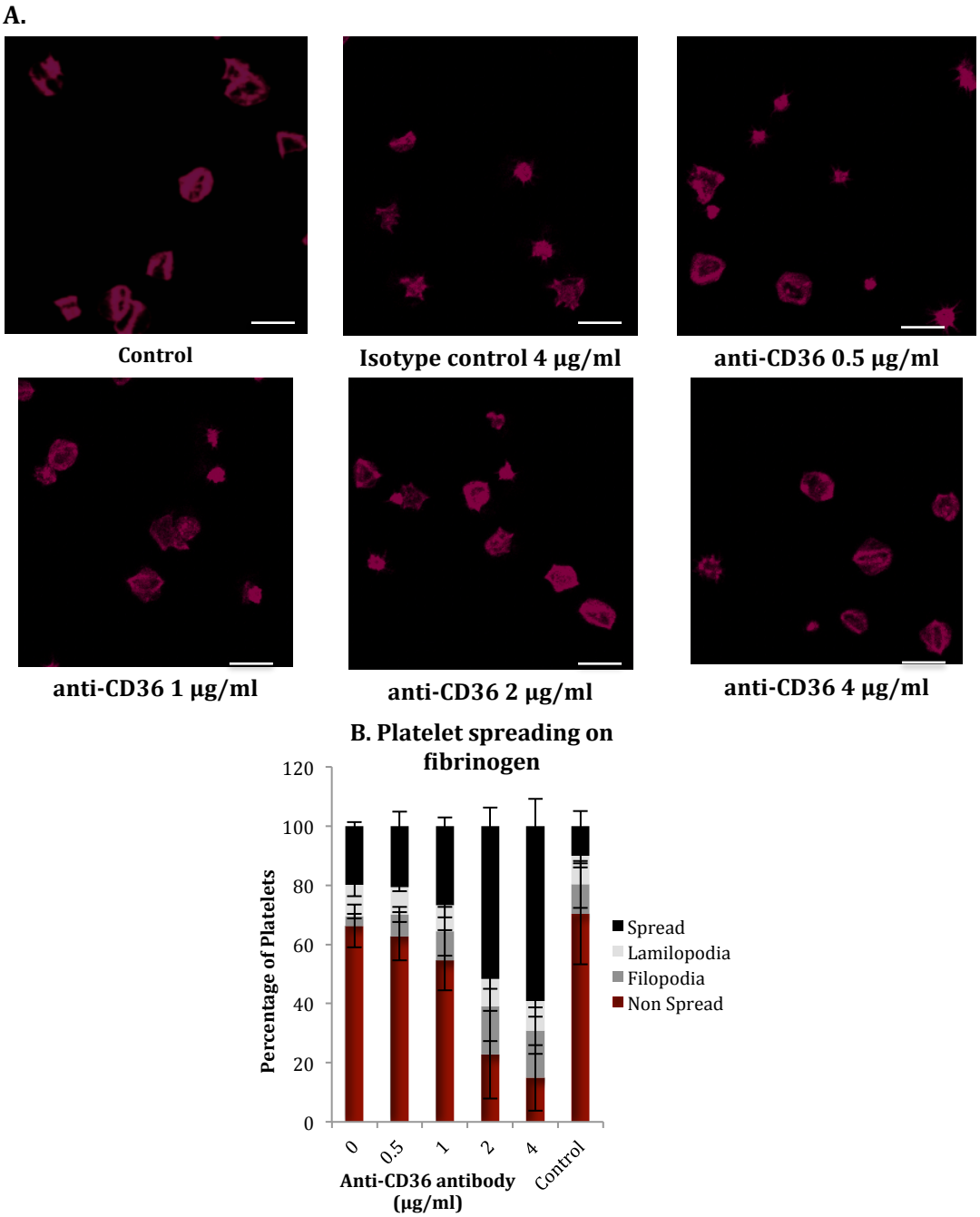

Figure 5.7: Inhibition of platelet spreading on fibrinogen by* S. aureus *lipoproteins is inhibited by neutralisation of CD36. A. *Images taken by fluorescence microscopy of platelet spreading on fibrinogen. Platelets were pretreated with different concentration of anti-CD36 antibody, then with* S. aureus ΔltaS *membrane proteins (8 µg/ml) and allowed to spread on fibrinogen for 45 min. Control represents untreated platelets allowed to spread on fibrinogen. Platelets labelled with philloidin 647.* ***B.*** *Data plotted as percentage of platelets spread, non-spread, platelets with filopodia and platelets with lamilopodia after allowing platelets incubated with different concentration of anti-CD36 and* S. aureus ΔltaS *membrane proteins. Control shows the data for isotype control antibody (4 µg/ml). Data represent mean values ± SEM. (Scale bars – 5µm (n=3).*

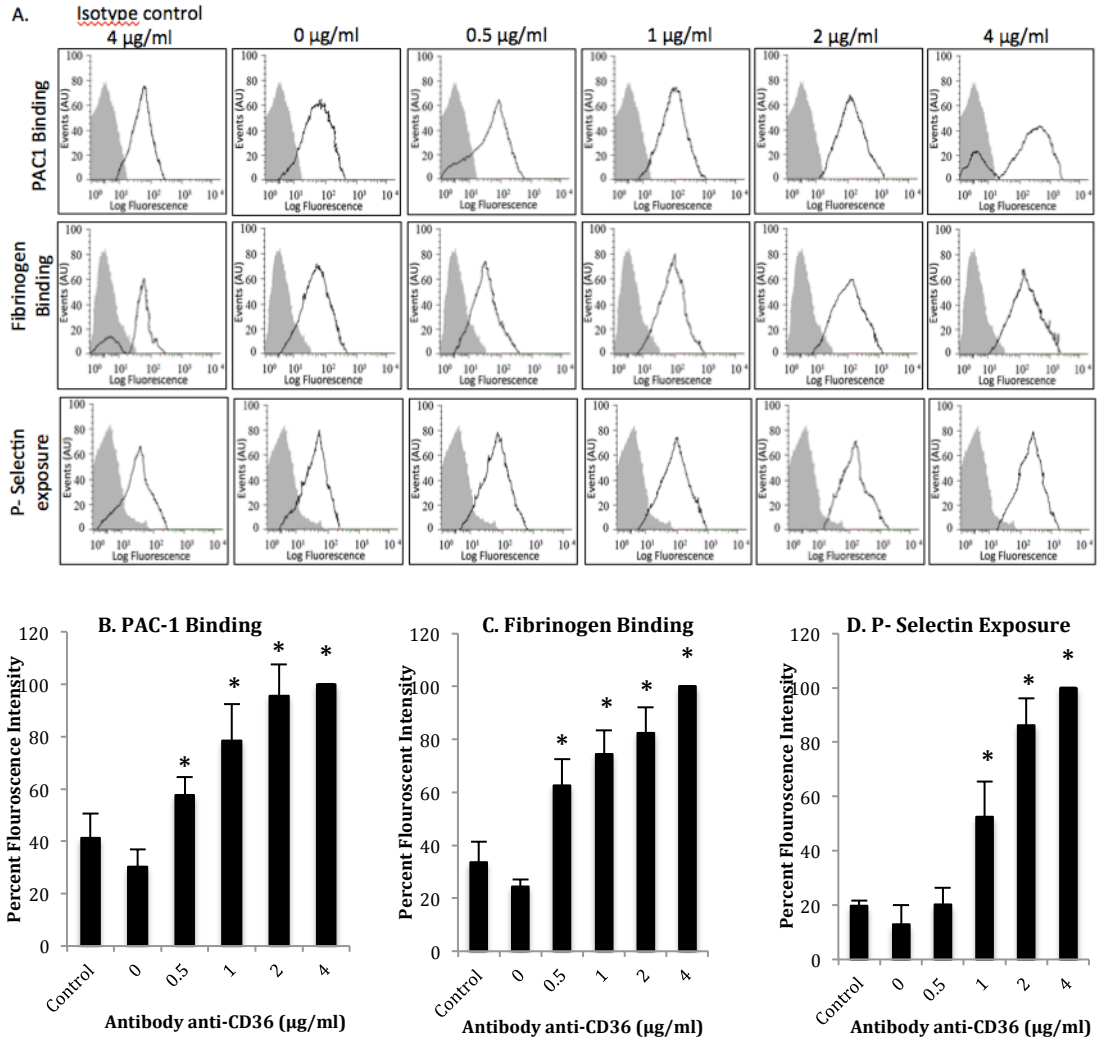

Figure 5.8: Inhibition of P- selectin expression, PAC1 and fibrinogen binding to platelets by* S. aureus *lipoproteins is inhibited on neutralisation of CD36. *Platelets were incubated with different concentration of anti-CD36 antibody, then with* S. aureus ΔltaS *membrane proteins (8 µg/ml) and stimulated with 0.1 µg/ml CRP-XL. Using flow cytometer activation of integrin αIIbβ3 was measured by analysing binding of PAC-1 and anti-fibrinogen antibodies whereas granule secretion was measured by looking at P- Selectin expression using anti CD62P antibody.* ***(A)*** *Histograms representing a typical experiment out of three performed with similar results, showing nullification of inhibition of PAC1binding, fibrinogen binding and P-Selectin exposure caused by* S. aureus *lipoproteins extract on incubation with anti CD36 antibody in a dose dependent. Data plotted as percentage of fluorescence intensity for binding of PAC-1 antibody* ***(A)****, anti fibrinogen antibody* ***(B)*** *and anti CD62P* ***(C)*** *to platelets. Control shows the data for isotype control antibody (4 µg/ml). Data represent mean values ± SEM. (n=3, *P<0.05)*

Conclusion

Lipoproteins can modulate platelet function (Rex *et al.* 2009). Known receptors for lipoproteins include toll like receptors (TLR's) (Brightbill *et al.* 1999) and scavenger receptors (Canton *et al.* 2013), many of which are present on platelets (Silverstein & Febbraio 2009; F Cognasse et al. 2005). *S. aureus* lipoproteins are essential pattern recognition molecules on the *S. aureus* cell surface (Wardenburg *et al.* 2006) and affect the function of immune cells via TLR2 (Li *et al.* 2008; Müller *et al.* 2010). Platelets express TRL2, and the triacylated lipoproteins Pam3CSK4 alters platelet function via TLR2 (Fälker *et al.* 2014), thus it was hypothesised to be a platelet surface receptor for *S. aureus* lipoproteins. Similarly high-density lipoproteins inhibit platelet activation via scavenger receptor B1 (SRB1) (Valiyaveettil *et al.* 2008). Due to inhibitory ability of *S. aureus* lipoproteins, SRB1 was considered to be another potential receptor for these lipoproteins. But the neutralisation of either receptor (Figure 5.4) as well as the mass spectrometry data (Figure 5.2) did not support a role for either.

On mass spectrometry analysis of platelet proteins co-precipitated with biotin tagged *S. aureus ΔltaS* membrane proteins, surface proteins co-precipitated included CD36, CD109, GPV, GPVI, GPIbα and β3. Among all of them, CD36 had the highest mass spectrometry prediction score and coverage (Figure 5.2) suggesting that it was the surface protein that was more strongly co precipitated with *S. aureus* lipoproteins. Co-immunoprecipitation of CD36 in the presence of biotinylated *S. aureus* membrane proteins showed that in comparison to the control, nearly all *S. aureus* membrane proteins co-precipitated with CD36 (Figure 5.3), thus confirming that it interacts with *S.*

aureus lipoproteins. Functional blocking of platelet surface SRB1 and TLR2 did not show any effect on *S. aureus* lipoproteins mediated inhibition of platelet activation thus co-precipitation assay was not performed, but there ability to interact with *S. aureus* lipoproteins cannot be ruled out.

It is been reported that plasma oxidised low-density lipoproteins cause activation of platelets via CD36 (Magwenzi *et al.* 2015). This was supported by our analysis as well. After neutralising CD36 on the platelet surface, platelet aggregation caused by plasma oxidised LDL was inhibited (Figure 5.5A). Pre-incubation of platelets with *S. aureus ΔltaS* membrane proteins also inhibited platelet aggregation caused by plasma oxidised LDL (Figure 5.5B). Thus suggesting that *S. aureus* lipoproteins might bind to CD36 and in turn block the access of plasma oxidised LDL, to cause any effect on platelets.

On neutralising CD36 on the platelet surface, inhibition of platelet aggregation (Figure 5.6), platelet spreading (Figure 5.7), integrin αIIbβ3 activation (Figure 5.8A,B) and platelet granule release (Figure 5.C) caused by *S. aureus* lipoproteins, was abolished. These data show that *S. aureus* lipoproteins not only interact with CD36 on the platelet surface, but interaction between them is also responsible for the observed inhibitory effect.

Chapter 6

Interaction of *S. aureus* lipoproteins with CD36 increases association of integrin β3 with CD36.

6.1. Introduction

Platelet CD36 is involved in inhibition of platelet activation by *S. aureus* lipoproteins. This 88kDa (Park 2014) major glycoprotein on platelet surface (Cserti-Gazdewich *et al.* 2009) is considered to be a receptor for thrombospondin (Wu 1995) and collagen (Nieswandt & Watson 2003). Binding of oxidized low-density lipoprotein to CD36 on the platelet surface leads to activation (Magwenzi *et al.* 2015). CD36 is also known to be receptor for fatty acids (Pepino *et al.* 2014), anionic phospholipids (Wagner 1996), and plasmodium falciparum malaria parasitized erythrocytes (Cserti-Gazdewich *et al.* 2009). Along with platelets, it is present on immune (Podrez *et al.* 2000), endothelial and epithelial cells (Park 2014).

Structurally CD36 has two trans membrane domains, both N- and C-terminals, embedded in membrane (Wagner 1996). It has short cytoplasmic tails and the majority of the protein is present in extracellular space (Wagner 1996). CD36 is heavily glycosylated at its large extracellular region (Hoosdally *et al.* 2009). There are ten known glycosylation sites present in this loop, of which nine are known to be modified during trafficking of the protein to the plasma membrane (Hoosdally *et al.* 2009). Defect in glycosylation block receptor trafficking, but glycosylation is not seen to be essential for ligand (oxidised LDL) recognition (Pepino *et al.* 2014). The extracellular domain of CD36 consists of three disulphide bridges at the carboxyl-terminal side (Gruarin *et al.* 1997).

Amino acids residues 87 to 279 form a loop and are speculated to be a part of binding pocket for CD36 ligands (Pepino *et al.* 2014) (Figure 6.1).

The intracellular portion of CD36 is smaller at the amino terminal but it is large enough at the carboxyl terminal end to allow binding and activation of protein kinases, leading to intracellular signalling (Silverstein *et al.* 2010). In many instances, interaction of ligands with CD36 causes activation of Src tyrosine kinases (Bull *et al.* 1994). In Cho cells and monocytes, stimulation of CD36 causes increase in intracellular Ca^{2+} level (Kuda *et al.* 2011). In platelets stimulation of CD36 by TSP-1 or oxidised LDL causes a Src kinases mediated increase in intracellular Ca^{2+} level (Nergiz-Unal *et al.* 2011). Stimulation of platelets by oxidised LDL causes Src kinases and Protein kinase C dependent phosphorylation and activation of NOX2. Activation of NOX2 increases the level of reactive oxygen intermediates (ROI) and dampening of cGMP mediated signalling (Magwenzi *et al.* 2015). Binding of TSP-1 also causes dampening of cGMP mediated signalling, thus promoting platelet activation (Isenberg *et al.* 2008). Overall, stimulation of platelet CD36 by TSP-1 or oxidised LDL leads to activation of platelets. Whereas upon blocking of CD36 platelets can be activated by other known agonists (Chapter 5), suggesting that signalling via CD36 is not essential for platelet activation.

Binding of *S. aureus* lipoproteins to platelet CD36 leads to inhibition of platelet activation by known physiological agonists (CRP-XL and Thrombin). In this chapter, possible mechanisms behind inhibition of platelet activation by *S. aureus* lipoprotein – CD36 interactions are evaluated.

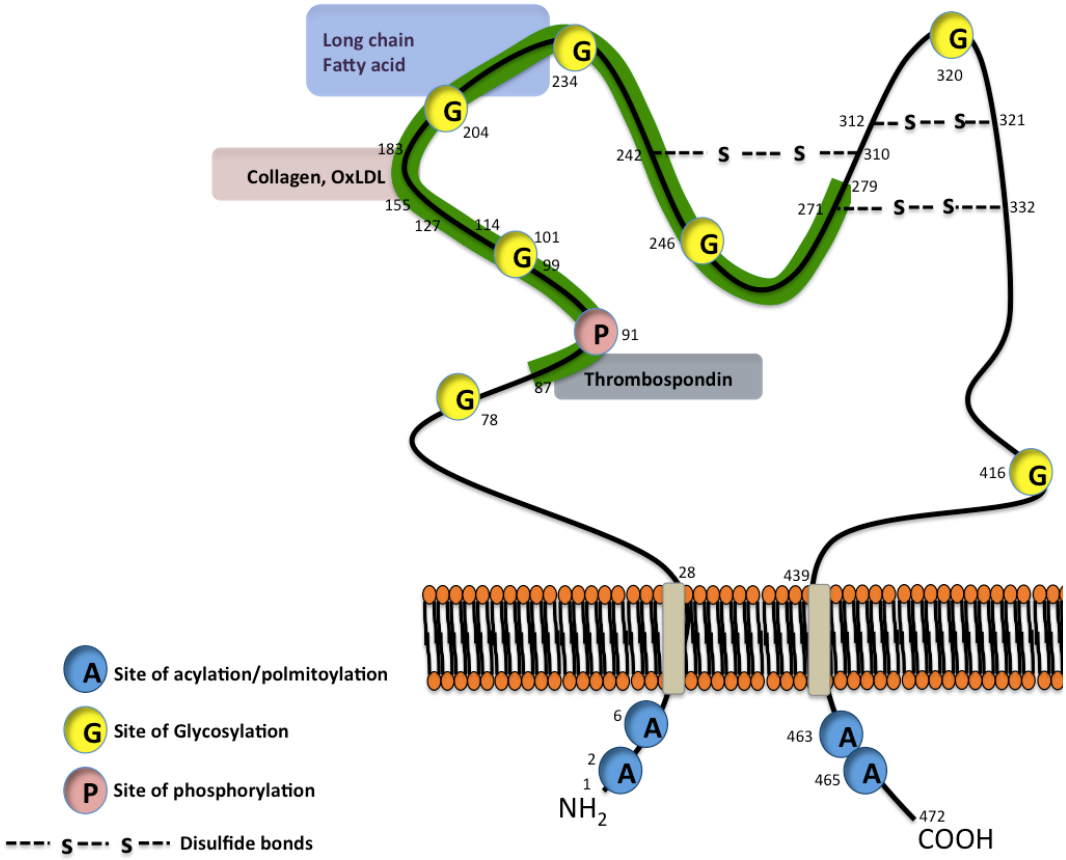

Figure 6.1: Proposed structure of CD36 molecule. *Proposed structure for CD36 showing sites for thrombospondin binding (amino acids 87 to 91), collagen and oxidised LDL binding (amino acid 155 to 183) and long chain fatty acid binding (amino acid 204 to 234). CD36 has three disulphide bonds at carboxyl side and six known glycosylation sites. Towards the cytoplasmic region CD36 is acylated at four sites, two from each side (carboxyl and amino). Carboxyl terminal end is longer then amino terminal. (Cayman Chemical™ Catalog)*

6.2: Results

6.2.1: *S. aureus* lipoproteins do not cause VASP phosphorylation.

Vasodilator-stimulated phosphoprotein (VASP) is associated with actin filaments and focal adhesions and is a regulator of actin dynamics (Aszodi et al. 1999). VASP is regulated by cAMP and cGMP dependent protein kinases (Li *et al.* 2003). Physiological platelet activation inhibitors like prostacyclin (PGI2) and nitric oxide (NO) increases the levels of cAMP and cGMP respectively (Pohl *et al.* 1994). The marker for increased levels of cAMP is phosphorylation of VASP at Ser239 and for cGMP is phosphorylation of VASP at Ser157 (Defawe *et al.* 2010).

Nitric oxide inhibits platelet function by binding to soluble guanylyl cyclase enzyme (sGC) that increases the levels of cGMP (Crane *et al.* 2005). Increased intracellular levels of cGMP affects multiple signalling pathways, such as cGMP-dependent receptor proteins, cGMP-regulated phosphodiesterases (PDE), and cGMP-dependent protein kinases (Wang *et al.* 1998). It also causes a decrease in intracellular calcium levels and inhibits integrin αIIbβ3 activation (Li *et al.* 2006). Upon increased in intracellular cGMP levels, the binding affinity of αIIbβ3 for fibrinogen is also decreased by cGMP-dependent inhibition of phosphoinositide 3-kinase activation (PI3K) and by cGMP-dependent phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) at Ser157 (Li *et al.* 2003).

Prostacyclin (PGI2) inhibits platelet function by binding to its G-protein coupled receptor on platelets (Klockenbusch *et al.* 1996). On binding, PGI2 causes a receptor induced signalling cascade, which induces adenylyl cyclase, leading to an increase in cAMP levels. Increase in cAMP level causes activation of

PKA that causes phosphorylation of several proteins including VASP (Ser239) (Raslan & Naseem 2015).

In order to test whether *S. aureus* lipoproteins cause VASP phosphorylation, platelet lysates were prepared by pre-incubating platelets with *S. aureus* membrane protein extracts. Stimulation of platelets by an agonist leads to different positive feedback mechanisms, which suppress the platelet inhibitory signalling (Isenberg *et al.* 2008). One such mechanism might be stimulation of CD36 that causes activation of NOX2 (NADPH oxidase 2), which increases the level of reactive oxygen intermediates (ROI) and thus dampens cGMP mediated inhibitory signalling (Magwenzi *et al.* 2015; Isenberg *et al.* 2008). In order to test whether *S. aureus* lipoproteins inhibit feedback mechanisms mediated through CD36 for agonist induced VASP phosphorylation, lysates were prepared of platelets pre-incubated with *S. aureus* membrane proteins and stimulated with CRP-XL. Western blotting for VASP phosphorylation at Ser239 and Ser157 (Figure 6.2A), showed no effect on VASP in presence of *S. aureus* lipoproteins. Nor was there any effect on agonist induced VASP phosphorylation on platelets pre-incubated with *S. aureus* lipoproteins (Figure 6.2). These data suggest that *S. aureus* lipoproteins do not induce increase in cAMP or cGMP levels in platelets. Also VASP phosphorylation was seen on both Ser239 and Ser157 sites without any difference on use of either PGI or NO donor (PAPA Nonoate) (Figure 6.2). Thus suggesting that there is no significant difference in VASP phosphorylation at these sites by either cAMP or cGMP mediated cascade.

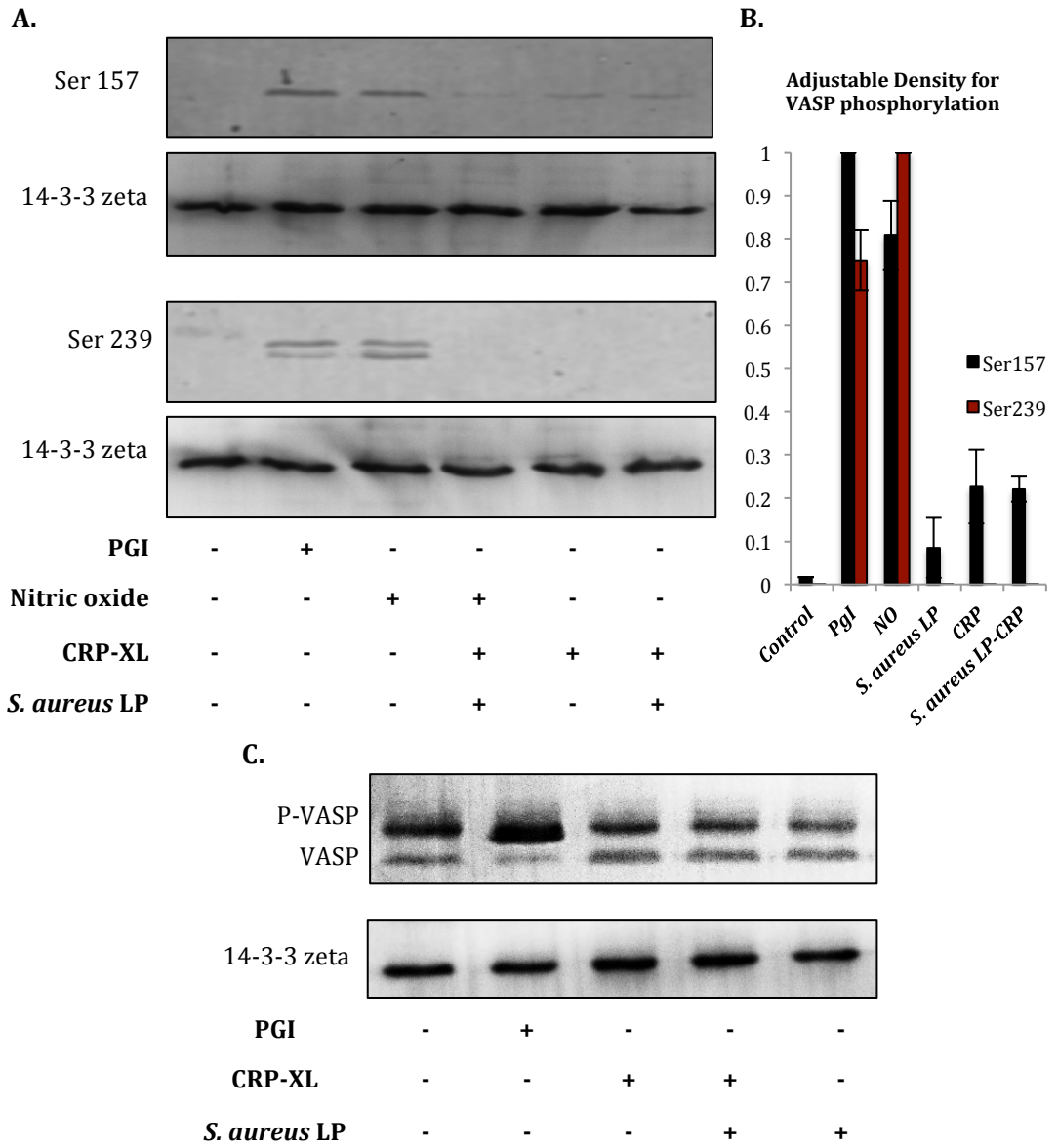

Figure 6.2:* S. aureus *lipoproteins do not cause or affect VASP phosphorylation.** ***A. *Representative western blot for VASP phosphorylation at Ser157 and Ser239 in platelet lysates made on incubation with PGI, PAPA NONOate (NO donor), CRP-XL and* S. aureus *lipoproteins. 14-3-3 zeta blotted as a loading control.* ***B.*** *Data plotted as adjustable density for VASP phosphorylation at Ser157 and Ser239 against 14-3-3 zeta loading control. Data represent mean values ± SEM. (n=3).* ***C.*** *Representative western blot for VASP, upper band showing VASP phosphorylation and lower band showing band for VASP in platelet lysates made on incubation with PGI, nitric oxide, CRP-XL and* S. aureus *lipoproteins. (n=3)*

6.2.2: *S. aureus* lipoproteins do not affect tyrosine phosphorylation or protein kinase C activity stimulated by CRP-XL.

Platelets express many receptor and non-receptor linked protein tyrosine kinases (PTK's) like Syk (Golden *et al.* 1986; Gao *et al.* 1997; Senis *et al.* 2015; Kralisz & Cierniewski 2000) and stimulation by agonists causes their activation. CRP-XL activates platelets in a manner similar to collagen (Polanowska-Grabowska *et al.* 2003). Collagen induced platelet activation causes tyrosine phosphorylation-dependent activation of phospholipase Cγ2 (PLCγ2) (Ezumi *et al.* 1998) and is the physiological platelet agonist that has been identified to induce phosphorylation of the FcRγ chain (Sundgren *et al.* 2011). The cytoplasmic tail of the FcRγ chain contains an immuno receptor tyrosine-based activation motif (ITAM), which is also responsible for attachment and activation of Syk (Liu *et al.* 2007). By looking at the tyrosine phosphorylation profile upon stimulation of platelets, the effect on platelet signalling can be assessed (Diaz-Ricart *et al.* 1999; Zurbano *et al.* 2003).

The protein kinase C (PKC) family of enzymes transduces numerous signals, promoting different stages of platelet activation (Yacoub *et al.* 2006). Agonist induced PKC activation occurs via secondary messengers DAG and inositol 1,4,5-trisphosphate (IP3) (Yoshida *et al.* 1992). Binding of CRP-XL to GPVI causes tyrosine kinase dependent activation of PLCγ2 (Jarvis *et al.* 2002). Activated PLCγ2 stimulates synthesis of DAG and inositol 1,4,5-trisphosphate (IP3). IP3 induces an increase in intracellular calcium levels by stimulating Ca^{2+} release from intracellular calcium stores (Li *et al.* 2010). Binding of Ca^{2+} to PKC promotes its tethering to membrane phosphotydylserine, thus allowing DAG to

bind. Binding of DAG causes a conformational change in PKC, allowing release of pseudo-substrate, empting the catalytically active pocket that causes phosphorylation of nearby substrates (Lenz *et al.* 2002). Activated PKC isoforms are involved in platelet granule release (Konopatskaya *et al.* 2011), thromboxane synthesis (Yacoub *et al.* 2006), platelet spreading (Harper & Poole 2010), integrin activation, aggregation and thrombus formation (Yacoub *et al.* 2006). The phosphorylation profile of PKC substrates is another marker for studying platelet signalling.

To examine the effect of *S. aureus* lipoproteins on platelet signalling, tyrosine phosphorylation and PKC substrate phosphorylation stimulated by CRP-XL, in platelets pre-treated with *S. aureus* membrane protein extracts, was analysed. There was no effect of *S. aureus* lipoproteins on tyrosine phosphorylation (Figure 6.3A) or PKC substrate phosphorylation (Figure 6.3B) in platelets. Also tyrosine phosphorylation and PKC substrate phosphorylation caused by stimulation of platelets by CRP-XL was unchanged on treatment with *S. aureus* lipoproteins. This suggests that *S. aureus* lipoproteins do not affect the initial platelet signalling caused upon activation by CRP-XL.

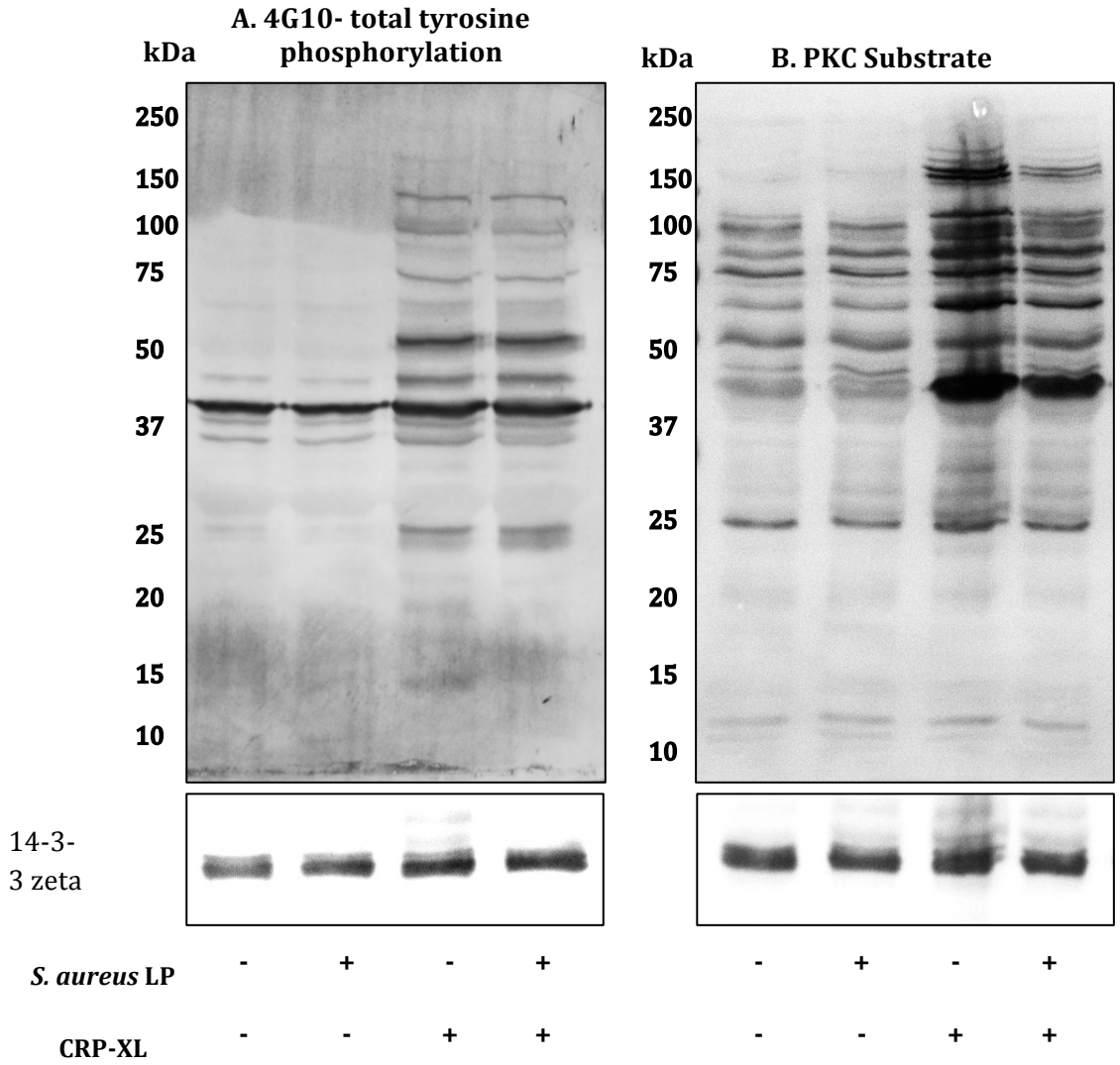

Figure 6.3: **S. aureus *lipoproteins do not cause or affect tyrosine phosphorylation or PKC substrate phosphorylation.*** ***A****. Representative western blot tyrosine phosphorylation (4G10) on stimulation with CRP-XL (30 sec),* S. aureus *lipoproteins (10 min) and CRP-XL (30 sec) on platelets pre-incubated with* S. aureus *lipoproteins (10 min).* ***B.*** *Representative western blot showing PKC substrate phosphorylation on stimulation with CRP-XL (30 sec),* S. aureus *lipoproteins (10 min) and CRP-XL (30 sec) on platelets pre-incubated with* S. aureus *lipoproteins (10 min). 14-3-3 zeta blotted as a loading control (n=3)*

6.2.3: *S. aureus* lipoproteins increase CD36 - integrin β3 association.

S. aureus lipoproteins inhibit platelet activation but do not cause VASP phosphorylation nor do they affect the initial tyrosine phosphorylation or PKC substrate phosphorylation caused by physiological agonists. From these signalling assays it can be speculated that the interaction between *S. aureus* lipoproteins and CD36 might not be causing any intracellular changes in platelets. To see what other molecules this interaction (*S. aureus* lipoproteins – CD36) might influence, mass spec data were analysed by string analysis (Jensen *et al.* 2009) (Figure 6.4). By focusing on CD36, it was seen that molecules predicated to interact with CD36 could be divided into three groups according to their cellular locations – intracellular, membrane bound or extracellular (Figure 6.4). From the signalling analysis, the interaction between CD36 and *S. aureus* lipoproteins does not alter intracellular proteins known to be involved in activation, so membrane bound proteins were considered as a possible mechanism of inhibition.

CD36 is known to physically associate with integrin $\alpha_3\beta_1$ and $\alpha_6\beta_1$ in human myeloma cells (Thorne *et al.* 2000) and CD9, αIIb, and β3 on human platelets (Miao *et al.* 2001). String analysis (Jensen *et al.* 2009) of the mass spectrometry data of platelet proteins associated with *S. aureus* lipoproteins showed the presence of β3, αIIb, GPIb and GPV associated with CD36 (Figure 6.4). In order to test whether *S. aureus* lipoproteins affect CD36-β3, CD36-αIIb and CD36-CD9 association, co-immuno precipitation assays were done for CD36 in the presence of membrane proteins from *S. aureus ΔltaS* and *S. aureus ΔltaS lgt* in resting platelets.

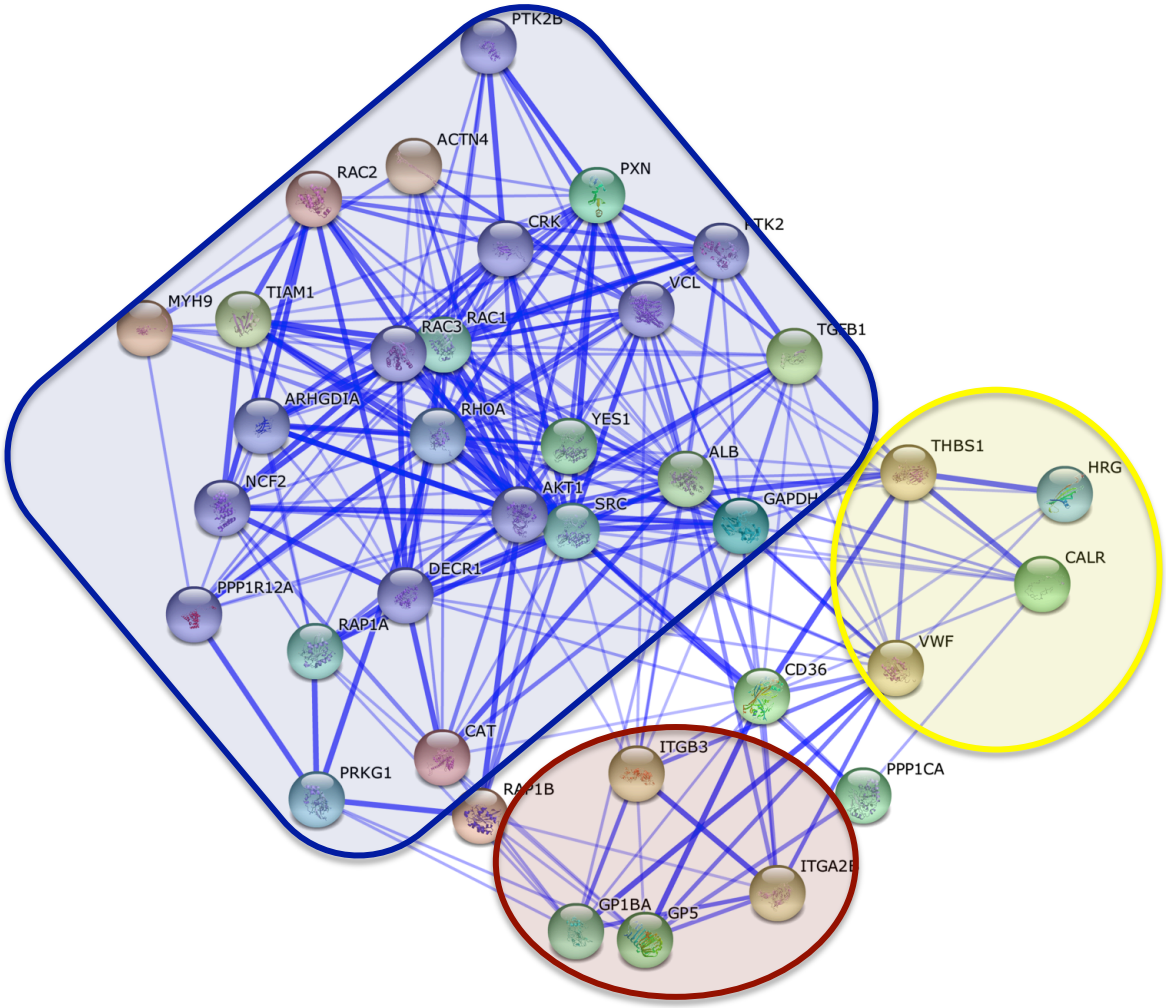

Figure 6.4: String analysis of mass spectrometry data. *String analysis on mass spectrometry data of platelet proteins co-precipitated with* S. aureus *lipoproteins. String network concentrated around CD36 and proteins showing interaction with it. Network divided into three sections depending upon position of proteins – intracellular (blue), extracellular (yellow) and membrane bound (red).*

Western blot of the co immuno-precipitation of CD36 samples for β3, αIIb and CD9 showed that the presence of *S. aureus ΔltaS* membrane proteins significantly increases integrin β3 co-precipitation with CD36 in comparison to samples with *S. aureus ΔltaS lgt* membrane proteins or control (Figure 6.5). The co-immunoprecipitation of CD36 varied, in most instances it was decreased in presence of *S. aureus* lipoproteins, which might be due to the less ability of anti-CD36 antibody to access its binding site, as in some instances *S. aureus* lipoproteins might occupy and hide it. The association of proteins binding to CD36 was calculated by adjusting the densities according to the relative CD36 pull down using Image-J. There was no detectable αIIb or CD9 on western blots for any of the samples including control (in absence of membrane proteins form *S. aureus* ΔltaS and *S. aureus* ΔltaS *lgt*), suggesting that the experimental conditions (washing) used might not be suitable to keep the association between this proteins and CD36 intact. This also suggests that interaction between αIIb or CD9 with CD36 might not be as strong as seen with β3. Presence of *S. aureus ΔltaS* membrane proteins increased the co-precipitation of β3 with CD36.

Agonist induced platelet activation was inhibited by *S. aureus* lipoproteins. To check whether increased CD36-β3 association due to *S. aureus* lipoproteins was changed in the presence of an agonist (CRP-XL), co immunoprecipitation for CD36 on CRP-XL stimulated platelets was done. At 1 μg/ml CRP-XL, inhibition of platelet aggregation by *S. aureus* lipoproteins was overcome. The same effect was observed on CD36-β3 association, on treatment of platelets with 1 μg/ml CRP-XL, the increased association between CD36-β3 caused by *S. aureus* lipoproteins was inhibited (Figure 6.6). At concentration of

0.1 μg/ml the increase in association between CD36-β3 was seen (Figure 6.6). Thus increased CD36-β3 association and inhibition of platelet activation might be correlated. Stimulation of platelets with CRP-XL only also slightly but significantly increases the CD36-β3 association, which suggests that this association might play role in platelet activation but in presence of S. aureus lipoproteins balance between this associations is affected suggesting it might play role in inhibition of platelet activation. Also, stimulation of platelets pre-incubated with *S. aureus* lipoproteins using a higher concentration of agonist leads to reduced CD36-β3 association and retraction of inhibition of aggregation. These data suggests that the increase in CD36-β3 association might be a key for understanding inhibition of platelet activation caused by *S. aureus* lipoproteins.

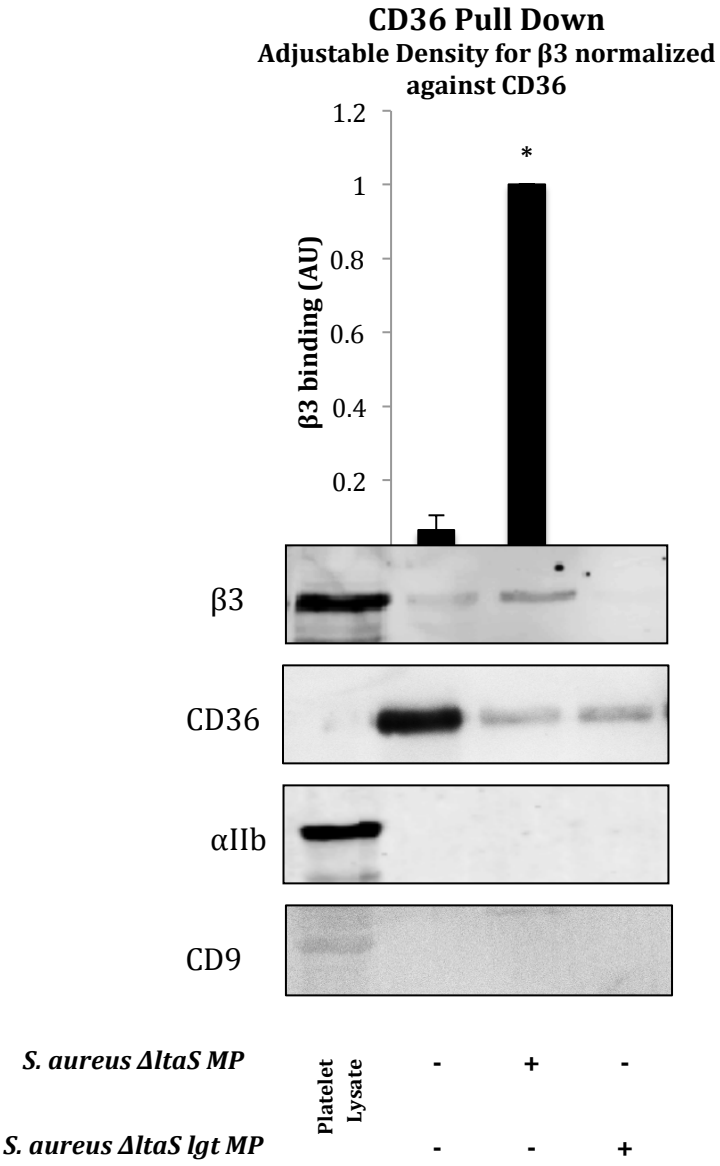

Figure 6.5: Co precipitation of integrin β3, αIIb and CD9 with CD36. *Resting platelet lysate treated with* S. aureus ΔltaS *membrane proteins and* S. aureus ΔltaS lgt *membrane proteins were co-immunoprecipitated for CD36 using anti-CD36 antibody and Protein G magnetic beads. Co-immunoprecipitation samples were western blotted for integrin β3, αIIb, CD36 and CD9. Adjustable density normalized against CD36 was calculated for integrin β3 using Image-J and represented as β3 binding. Data represent mean values ± SEM. (n=3, *P<0.01)*

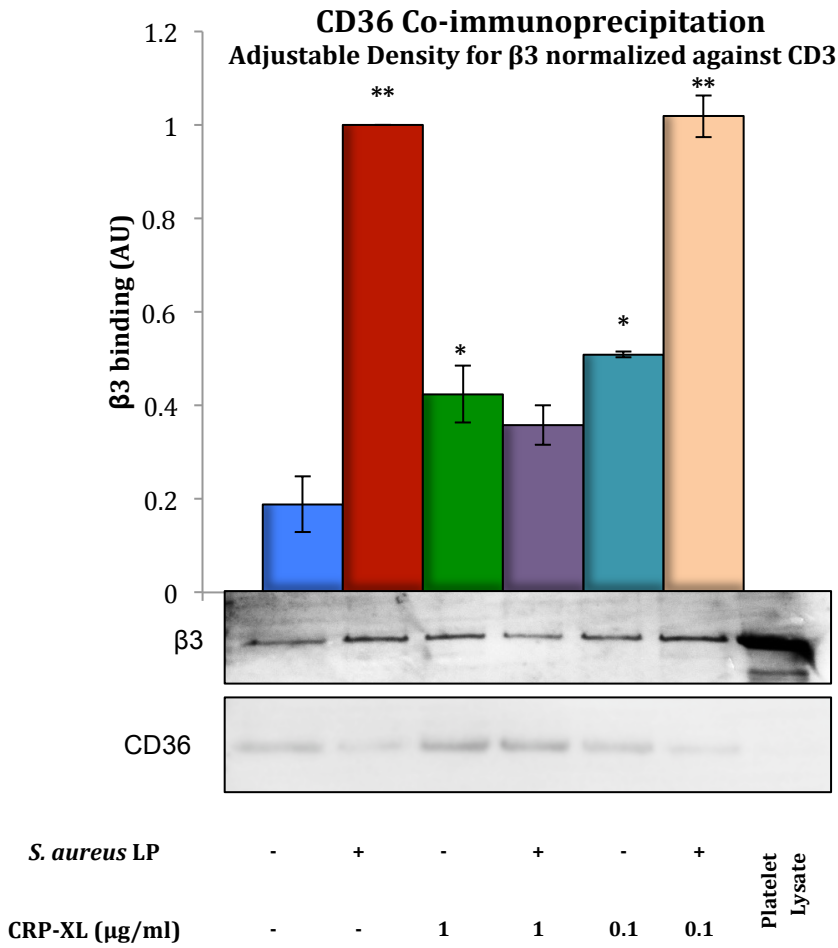

Figure 6.6: Co-precipitation of integrin β3 with CD36. *Platelet treated with* S. aureus ΔltaS *membrane proteins and different concentrations of CRP-XL were lysed and co-immunoprecipitated for CD36 using anti-CD36 antibody and Protein G magnetic beads. Co-immunoprecipitation samples and platelet lysate as a control were western blotted for integrin β3 and CD36. Adjustable density normalized against CD36 was calculated for integrin β3 using Image-J and represented as β3 binding. Data represent mean values ± SEM. (n=3, *P<0.05, **P<0.01).*

6.2.4: The interaction between *S. aureus* lipoproteins and CD36 is responsible for the increased association between integrin β3 and CD36.

In the presence of *S. aureus* lipoproteins, the association between CD36-β3 was increased significantly. *S. aureus* lipoproteins bind to CD36 and inhibition of platelet activation occur. To analyze whether the interaction between *S. aureus* lipoproteins and CD36 was responsible for increased association between CD36-β3, binding of biotinylated *S. aureus* lipoproteins to CD36 was blocked by an anti-CD36 antibody. After co-precipitating *S. aureus* lipoproteins using streptavidin beads, the reaction mixture was examined for CD36 and β3. Control showed the presence of β3 suggesting direct interaction between this protein and streptavidin beads. The plot (Figure 6.7) represents the relative densities for the bands obtained, as it was not possible to calculate the adjustable densities against the *S. aureus* lipoproteins due to the presence of numerous bands. Even though the normalization was not possible but from the relative band densities it was observed that, as the concentration of anti-CD36 antibody was increased, the amount of CD36 as well as β3 was significantly decreased (Figure 6.7). Thus inhibition of the interaction between *S. aureus* lipoproteins and CD36, also inhibited the association between CD36 and β3. These data suggest that the interaction between *S. aureus* lipoproteins and CD36 is responsible for increase in association between CD36-β3.

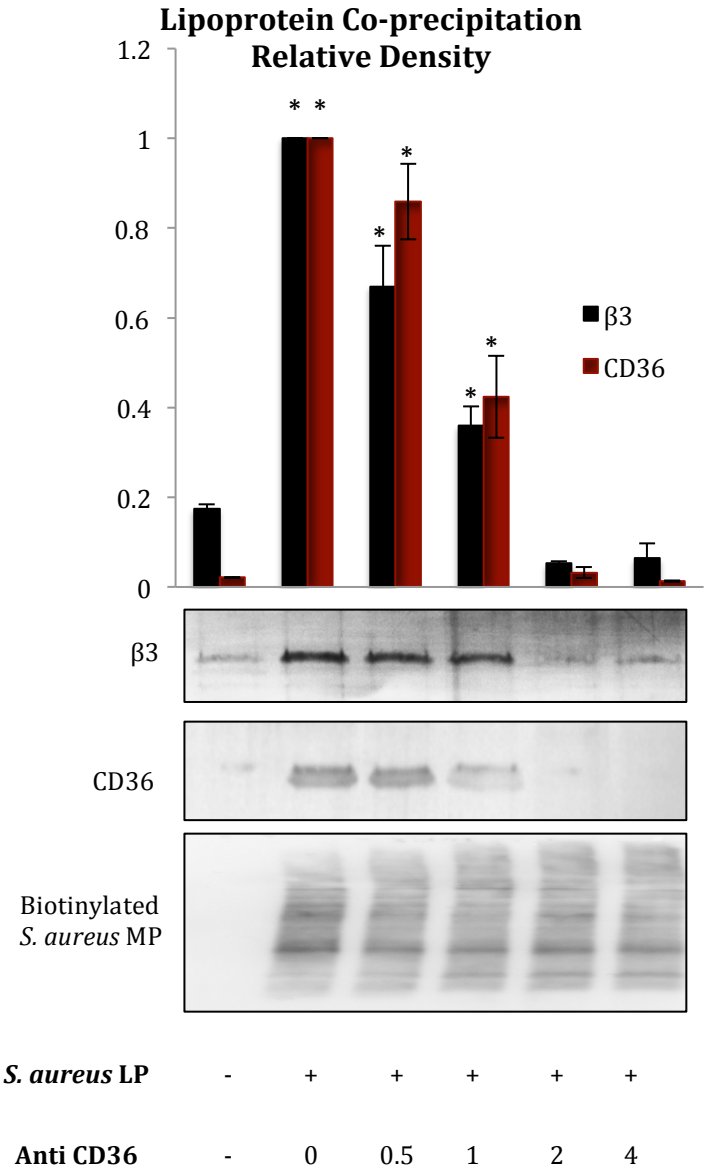

Figure 6.7: Role of the CD36* – S. aureus *lipoproteins interaction in increased CD36-β3 association. *Platelet treated with biotinylated* S. aureus ΔltaS *membrane proteins and different concentrations of anti-CD36 were lysed and co precipitated for biotinylated* S. aureus *lipoproteins using streptavidin beads. Co precipitation samples were western blotted for integrin β3, CD36 and* S. aureus *lipoproteins. Relative densities for CD36 and integrin β3 using Image-J were calculated. Data represent mean values ± SEM. (n=3, *P<0.05 vs control).*

6.2.5: Interaction between *S. aureus* lipoproteins and CD36 do not affect FcγRIIa association.

Fc receptors are surface proteins that have binding affinity for the Fc portion of an antibody (Ramsland *et al.* 2011). Fc receptors play a significant role in immune and inflammatory responses and are expressed on macrophages (Dorrington 1976), neutrophils (Repp *et al.* 1991), natural killer cells, B-lymphocytes (Perussia *et al.* 1984), mast cells (Malbec & Daëron 2007) and platelets (Shido *et al.* 1995). FcγRIIa is the Fc receptor present on platelets (Shido *et al.* 1995) and is known to play a role in the internalization of IgG (Worth *et al.* 2006), immune mediated thrombocytopenia (McKenzie *et al.* 1999) and platelet activation by contributing to out-side in signalling (Zhi *et al.* 2013). FcγRIIa is also an essential component in *S. aureus* mediated platelet activation (Fitzgerald, Loughman, *et al*. 2006).

Mouse platelets lack the FcγRIIa receptor (Zhi *et al.* 2013). Pre incubation of mouse platelets with *S. aureus* lipoproteins does not affect their activation by physiological agonists (Chapter 4). Also FcγRIIa receptor plays role in amplification of platelet activation signalling mediated via integrin αIIbβ3 (Boylan *et al.* 2008). There is no reported evidence of direct interaction between FcγRIIa and integrin αIIbβ3, but *S. aureus* lipoproteins increases the CD36-β3 association and FcγRIIa is significant in *S. aureus* mediated platelet activation (Arman *et al.* 2014) that makes it important to explore its involvement. In order to investigate if *S. aureus* lipoproteins – CD36 interaction affect or cause any interaction with FcγRIIa, co-immuno precipitation for FcγRIIa was done on platelets incubated with *S. aureus ΔltaS* membrane proteins and/or CRP-XL.

Western blotting the co-immuno precipitated samples for integrin β3 and CD36, it was observed that none of them were co-precipitated with FcγRIIa and presence of *S. aureus ΔltaS* membrane proteins and/or CRP-XL did not cause any of them to associate (Figure 6.8). These data suggests that there might be no physical level association between integrin β3 and FcγRIIa or CD36 and FcγRIIa but its involvement in inhibition of platelet activation caused by *S. aureus* lipoproteins cannot be ruled out.

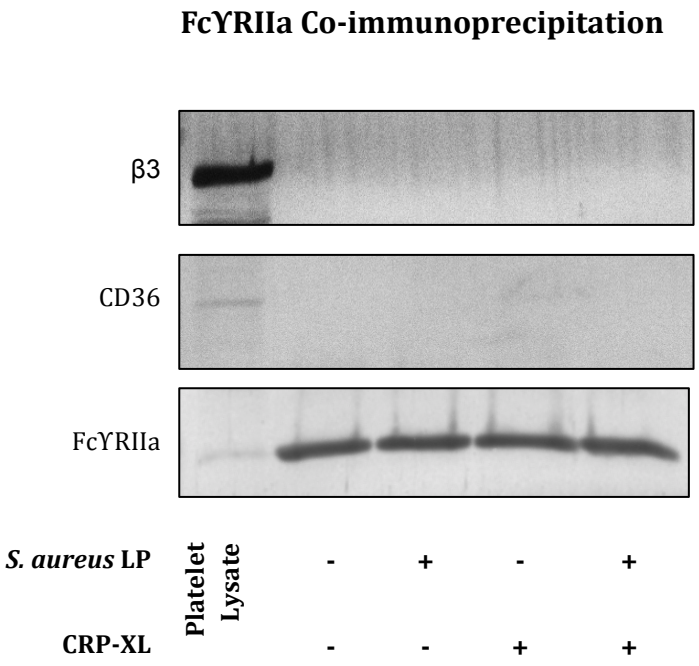

Figure 6.8: Co-precipitation of integrin β3 and CD36 with FcYRIIa*. Platelets pre-incubated with* S. aureus ΔltaS *membrane proteins and/or CRP-XL were lysed and co-immunoprecipitated for FcYRIIa using anti CD32 antibody (anti Fc receptor) and protein G magnetic beads. Co-immunoprecipitation samples were western blotted for integrin β3, CD36 and FcYRIIa. (n=3)*

Conclusion

The interaction of ligands with CD36 typically activates platelets (Isenberg *et al.* 2008; Ghosh *et al.* 2011; Nergiz-Unal *et al.* 2011). However it is shown in this work that inhibition of platelet activation can also occur via CD36. Stimulation of platelet CD36 leads to activation of kinases (Bull *et al.* 1994) and modulation of feedback mechanisms during platelet activation (Magwenzi *et al.* 2015). To test the effect of kinase activity on platelets treated with *S. aureus* lipoproteins, tyrosine phosphorylation and PKC substrate phosphorylation were examined. The phosphorylation profile showed that there was no effect on protein phosphorylation caused by agonists on platelets pre-incubated with *S. aureus* lipoproteins (Figure 6.3). Thus *S. aureus* lipoproteins are able to inhibit platelet activation without affecting protein phosphorylation and initial platelet signalling.

Normal haemostasis is maintained by controlling platelet activation. Under physiological conditions, platelet activation is inhibited by two major vasodilators released by endothelial cells and platelets themselves- nitric oxide and prostacyalin (PGI2) (Rivera *et al.* 2009). Both control the VASP proteins that regulate the actin dynamics and integrin activation in platelets preventing platelet activation (Aszodi *et al*. 1999). CD36 plays a part in a feedback mechanism by increasing the ROIs that control nitric oxide levels, thus decreasing intracellular cGMP concentrations and promote platelet activation (Magwenzi *et al.* 2015). To explore whether CD36 - *S. aureus* lipoprotein interactions affect this modulatory ability of CD36, VASP phosphorylation caused in platelets treated with *S. aureus* lipoproteins was assessed. *S. aureus*

lipoproteins had no effect on resting platelets VASP phosphorylation nor did it have any effect on agonist induced VASP phosphorylation. This suggests that inhibition of platelet activation by *S. aureus* lipoproteins does not involve increase in cyclic guanosine monophosphate (cGMP) or cyclic adenosine monophosphate levels (cAMP) and subsequent phosphorylation of VASP.

The thrombus under flow assay and calcium release assay showed that initial agonist induced thrombus formation and intracellular calcium increase is not affected in the presence of *S. aureus* lipoproteins (Chapter 4). The signalling data obtained supports that observation and suggests that *S. aureus* lipoproteins inhibit platelet activation by some other mechanism.

CD36 can associate with platelet integrins (Miao *et al.* 2001). To see whether this association is affected by *S. aureus* lipoproteins, co-immuno – precipitation of CD36 on platelets treated with *S. aureus* lipoproteins was done. It showed that in the presence of *S. aureus* lipoproteins, association between CD36 and platelet integrin β3 was significantly increased and this increase was dependent on interactions between *S. aureus* lipoproteins and CD36. Integrin β3 is an important component of αIIbβ3, a major integrin on platelets. On incubation of platelets with *S. aureus* lipoproteins, increased PAC-1 binding (the antibody that binds to active from of αIIbβ3) caused by agonists was inhibited (Chapter 4). Activation of αIIbβ3, leads to its clustering, followed by binding of ligands to the clustered αIIbβ3 (Gaul *et al.* 2015). One of the major ligands for active αIIbβ3 is fibrinogen. In the presence of *S. aureus* lipoproteins agonist induced fibrinogen binding and spreading on fibrinogen was reduced (Chapter 4). These results again suggest that *S. aureus* lipoproteins inhibit αIIbβ3

activation. Direct physical level associations between CD36 and integrin β3 in the presence of *S. aureus* lipoproteins might affect the agonist induced conformational change and activation of αIIbβ3.

It is been reported that CD36 associates with CD9, αIIb, and β3 on human platelets (Miao *et al.* 2001). Mass spectrometry data showed the presence of αIIb interacting with *S. aureus* lipoproteins but it did not co – precipitate with CD36 in the presence or absence of *S. aureus* lipoproteins. In the case of CD9, mass spectrometry data and co-immunoprecipitation assay did not show any direct association of CD36 with CD9. Also due to differences in the effect of *S. aureus* lipoproteins on mice and human platelets, FcγRIIa was thought to be involved, as mice platelets lacks FcγRIIa. But co-immunopreciptiation assays showed no association of FcγRIIa with integrin β3 and CD36 in absence or presence of S. *aureus* lipoproteins.

The increase in association between integrin β3 and CD36 caused by *S. aureus* lipoproteins is not seen in the presence of higher concentrations of agonist (Figure 6.6). Earlier inhibitory studies have shown that on increasing the concentration of agonist, inhibition of platelet aggregation caused by *S. aureus* lipoproteins is inhibited (Chapter 4). The fact that high concentration of agonist inhibit the increased β3 - CD36 interaction, which in-turn abrogate inhibitory effect of *S. aureus* lipoproteins suggests that this association might be the key mechanism behind the inhibition of platelet activation caused by *S. aureus* lipoproteins.

Chapter 7
Discussion

Under normal physiological conditions, platelets are one of the most abundant cells in human blood. Therefore during bacteraemia, platelets can be speculated to be the first host cell that comes in contact with pathogen (Cox *et al*. 2011). Numerous bacteria can modulate platelet function (Fitzgerald *et al*. 2006). A role for platelets in host defence has not been clearly elucidated but platelets are known to play a part in immune function by secreting cytokines that recruit immune cells (Hamzeh-Cognasse *et al*. 2015) and by directly interacting with immune cells (Etulain *et al*. 2015), aiding their function. Thus, by modulating platelet function, bacteria can regulate this platelet response which might aid in its survival in host.

S. aureus is one of the major pathogenic bacteria which has been extensively studied for its ability to modulate platelet activity. Numerous *S. aureus* surface proteins are known to interact with platelets either directly (Miajlovic *et al*. 2010) or via a plasma protein bridge (Fitzgerald, Loughman, *et al*. 2006; Loughman *et al*. 2005) and cause their activation. Along with surface proteins, exoproteins like α-toxins (Hugo *et al*. 1988), Efb (Shannon & Flock 2004) and molecules like LTA (Waller *et al*. 2013) are also known to modulate platelet function.

S. aureus lipoproteins are some of the surface proteins that are recognised by the host immune system and play role in eradication of *S. aureus* infection by the host (Wardenburg *et al*. 2006). *S. aureus* lipoproteins also show binding

ability to platelets and once in the extracellular milieu can inhibit platelet activation.

7.1. Binding of *S. aureus* lipoproteins to platelets

S. aureus mutants lacking lipidation of proteins had a significantly longer aggregation lag time and showed reduced binding ability to platelets. The *S. aureus* lipoproteins biogenesis process involves, acylation of pro-lipoproteins anchored in the cell membrane via a signal peptide by lipoprotein diacylglyceryl transferase (Lgt) and then removal of the signal peptide by lipoprotein signal peptidase (Lsp) (Nakayama *et al*. 2012). The acyalted portion of lipoproteins inside the cell membrane functions as an anchor once the signal peptide is truncated (Blanc *et al*. 2013). In *S. aureus lgt* mutants a greater amount of non–acylated pro-lipoproteins are expressed on the bacterial surface and as well released in supernatant (Stoll *et al*. 2005). *S. aureus* lipoproteins lacking acylation are not recognized by host immune cells and can persist longer (Bubeck Wardenburg *et al*. 2006). By this observation it can be speculated that there is a direct interaction between *S. aureus* lipoproteins and immune cells, as also seen with platelets. Even though the lipidated portion of lipoproteins is embedded in the cell membrane, it plays a key role in the direct interaction of *S. aureus* with host immune cells (Blanc *et al*. 2013) and platelets. The absence of *S. aureus* lipoproteins affected the platelet aggregation lag time, but did not affect the percentage aggregation of platelets caused by *S. aureus*. Thus suggesting that lipoproteins contribute to interaction between *S. aureus* and platelets but does not significantly alter the platelet activation ability of *S. aureus* when present on the bacterial surface.

S. aureus releases different molecules by secretion and sheading from the cell envelope. One class of molecules released by *S. aureus* into the extracellular milieu are lipoproteins (Stoll *et al*. 2005). The extracted *S. aureus* membrane proteins showed an ability to bind platelets, whereas in comparison to wild type, membrane proteins from *S. aureus lgt* did not. This observation support the earlier finding that lipidaiton of *S. aureus* lipoproteins, whether on the surface or in suspension, is essential in binding to platelets.

7.2. Inhibition of platelet activation by *S. aureus* lipoproteins

Incubation of platelets with membrane proteins extracted from *S. aureus ΔltaS* inhibited platelet activation whereas membrane proteins extracted from *S. aureus lgt ΔltaS* did not show any effect. The TX-114 phase extraction method was used to extract hydrophobic membrane proteins (Li *et al*. 2008; Asanuma *et al*. 2011; Cockayne *et al*. 1998) and on using this method the extract of lipoproteins from *S. aureus* showed contamination with lipotechioc acid (LTA). *S. aureus* LTA inhibits platelet activation by increasing cAMP levels via the PafR receptors (Waller *et al*. 2013). To avoid LTA contamination, *S. aureus ΔltaS* was used which did not produce LTA. For an appropriate control double mutant, *S. aureus lgt ΔltaS* was constructed that was deficient of LTA and lacked lipidation of *S. aureus* lipoproteins.

Stimulation of platelets by a physiological agonist causes numerous signalling and sequential events leading to platelet activation (Watson & Gibbins 1998; Sambrano *et al*. 2001). Sustained increases in the level of intracellular calcium are essential for activity of many enzymes (kinases) that cause intracellular signalling (Li *et al*. 2010), integrin activation (Tsuboi 2002), platelet

spreading (Naik & Naik 2003) and thrombus formation. Initial signalling stimulated by a physiological agonist on platelets pre-incubated with *S. aureus ΔltaS* membrane proteins was not significantly different from controls. Also sustained increases in intracellular calcium levels caused by integrin activation in platelets were inhibited on preincubation with *S. aureus ΔltaS* membrane proteins. The thrombus formation kinetics on incubation with *S. aureus ΔltaS* membrane proteins showed that initial platelet adhesion to the collagen coated surface was not affected but a later stable thrombus was not formed. This suggests that the later stages of platelet activation are inhibited on pre-incubation of platelets with *S. aureus ΔltaS* membrane proteins without affecting initial adhesion or signaling events caused by known physiological platelet agonists. There was no effect seen of membrane proteins extracted from *S. aureus lgt ΔltaS* thus suggesting role of *S. aureus* lipoproteins in this inhibition.

S. aureus lipoproteins inhibit PAC-1 binding i.e. activation of integrin αIIbβ3 and binding of fibrinogen, both events necessary for platelet-platelet binding and formation of an aggregate (Bennett 2005). Thus on pre-incubation of platelets with *S. aureus ΔltaS* membrane proteins platelet aggregation was inhibited. Stable thrombus formation at an injury site is the result of interaction between platelets and extracellular matrix proteins that leads to activation of platelets (Kroll *et al*. 1991) and release of granules (Whiteheart 2011) that aid in recruitment and activation of additional platelets. On release of granules, platelets P-selectin molecules are expressed on the activated platelet surface (Koedam *et al*. 1992). On pre-incubation of platelets with *S. aureus ΔltaS* membrane proteins P-selectin exposure caused by physiological agonists was

inhibited, which suggests inhibition of granule release. Lack of granules leads to inhibition of platelet recruitment and stable platelet – platelet interaction, causing unstable thrombus formation (Whiteheart 2011). Pre incubation of platelets with *S. aureus ΔltaS* membrane proteins also causes inhibition of platelet spreading on fibrinogen. Binding of activated integrin αIIbβ3 to fibrinogen causes out-side in signalling which leads to cytoskeletal rearrangements in platelets leading to platelet spreading (Lee *et al*. 2012). Inhibition of platelet spreading also suggests inhibition of integrin αIIbβ3 by *S. aureus ΔltaS* membrane proteins. Control of membrane proteins extracted from *S. aureus lgt ΔltaS* did not show any effect on platelet activation suggesting involvement of *S. aureus* lipoproteins in inhibition of integrin αIIbβ3.

7.3. Inhibition of platelet activation by *S. aureus* lipoproteins is mediated via CD36.

S. aureus lipoproteins are recognized by host immune cells via TLR-2, which leads to signalling and host response against *S. aureus* (Tawaratsumida *et al*. 2009; Müller *et al*. 2010; Kang *et al*. 2015). Platelets express TRL-2 (Cognasse *et al*. 2005) but mass–spectrometry analysis of platelet proteins co-precipitated with *S. aureus* lipoproteins did not show TLR-2 to be present. This absence of TLR2 might be due to the low abundance of TLR2 molecules present on platelets (Cognasse *et al*. 2005). On neutralization of TRL2 on the platelet surface, inhibition of platelet activation caused by *S. aureus* lipoproteins was not abrogated. Stimulation of platelet TRL2 causes an activation response in platelets (Fälker *et al*. 2014) whereas *S. aureus* lipoproteins caused inhibition.

These findings indicated that the effect of *S. aureus* lipoproteins on platelet function is probably not via TLR-2.

The functional relationship of scavenger receptors and modified lipoproteins is well established in atherosclerosis and haemostasis (Canton *et al*. 2013). Scavenger receptors are known to recognise and remove modified lipoproteins (Zingg *et al*. 2000). Platelets express scavenger receptors like CD36, SRB1, CD68 and LOX1 (Valiyaveettil & Podrez 2009). Oxidised high-density lipoprotein, on interaction with SRB1, inhibits platelet activation (Valiyaveettil, Kar, Ashraf, Byzova, Febbraio & E. a Podrez 2008). But neither mass spectrometry data from platelet proteins co-precipitated with *S. aureus* lipoproteins, nor platelet SRB1 neutralisation showed that *S. aureus* lipoproteins bind SRB1 or functionally alter platelet function via it. Also other than CD36, mass spectrometry data from platelet proteins co-precipitated with *S. aureus* lipoproteins did not show the presence of any other scavenger receptor. On blocking CD36, inhibition of platelet activation caused by *S. aureus* lipoproteins was abrogated, indicating that CD36 not only binds *S. aureus* lipoproteins, but also was responsible for functional effect caused by them.

CD36 is also known as a thrombospondin receptor (Nergiz-Unal *et al*. 2011; Silverstein & Febbraio 2009; Bull *et al*. 1994). The interaction of CD36 with its ligands causes production of reactive oxygen intermediates, neutralizing NO and thus promotes platelet activation via this positive feedback mechanism (Magwenzi *et al*. 2015). The presence of *S. aureus* lipoproteins that binds and occupy CD36 on platelets might prevent the interaction of thrombospondin will thus inhibit the positive feedback mechanism during activation of platelets.

Platelet activation caused by the other known physiological agonists was inhibited by interaction between *S. aureus* lipoproteins and CD36. Platelet activation and signalling caused via CD36 upon interaction with oxidised LDL is known (Magwenzi *et al*. 2015; Silverstein *et al*. 2010). The interaction of CD36 with ligands leads to activation of Syk kinases and subsequent phosphorylation causes platelet activation (Silverstein *et al*. 2010). However, the presence of *S. aureus* lipoproteins did not affect the tyrosine and VASP phosphorylation caused by CRP-XL, suggesting that *S. aureus* lipoproteins did not cause or affect the initial signalling. Focusing around proteins interacting with CD36 in Mass spectrometry data from platelet proteins co-precipitated with *S. aureus* lipoproteins, I found three clear divisions (extracellular, intracellular and membrane proteins) in the interaction patterns. In the case of extracellular proteins, it can be proposed that their normal interaction with CD36 in the presence of *S. aureus* lipoproteins will be altered and thus won't cause their normal physiological effect on platelets. From the signalling data obtained it can be speculated that the intracellular proteins were not affected by CD36- *S. aureus* lipoproteins interaction. Thus the membrane proteins interacting with CD36 in the presence of *S. aureus* lipoproteins might be the main contributing factor in inhibition of platelet activation.

CD36 associates with platelet membrane proteins β3, αIIb and CD9 (Miao *et al*. 2001); the functional significance of these interactions is not yet established. Co-immunoprecipitation analysis showed that the presence of *S. aureus* lipoproteins affects the CD36-β3 association. During platelet activation, conformational changes in integrin αIIbβ3 change its affinity state from low to

high, allowing it to bind its ligands which leads to signalling events and platelet activation (Mehrbod *et al*. 2013). Activation of integrin αIIbβ3 is proposed to occur by a clasping/unclasping mechanism (Ma *et al*. 2007). The extracellular region of αIIb and β3 are clasped with each other until the transmembrane region (Figure 7.1A) (Kim *et al*. 2009). Binding of talin to the cytoplasmic region of β3 initiates an unclasping event that leads to conformational changes in the transmembrane, and subsequently extracellular domains of αIIbβ3 (Kahner *et al*. 2012). The activation phenomenon is seen to be reversible, as on detachment of talin by other proteins (β3 - endonexin) competing for the same site causes return of αIIbβ3 to inactive conformation (Kashiwagi *et al*. 1997). The equilibrium between proteins responsible for clasping and unclasping of αIIb and β3 is speculated to be significant in deciding in fate of αIIbβ3 activation (Figure 7.1 A) (Ma *et al*. 2007).

Association of other surface molecules with platelet integrin αIIbβ3 occurs (Miao *et al*. 2001), but the manner in which this association might affect the equilibrium of integrin activation is unknown. It can be speculated that an increase in CD36-β3 association in presence of *S. aureus* lipoproteins might affect the equilibrium and block the normal transition of integrin αIIbβ3 from inactive to active form caused by stimuli. Inhibition of platelet activation by *S. aureus* lipoproteins might be due to changes in CD36-β3 association (Figure 7.1B), but further evaluation is necessary.

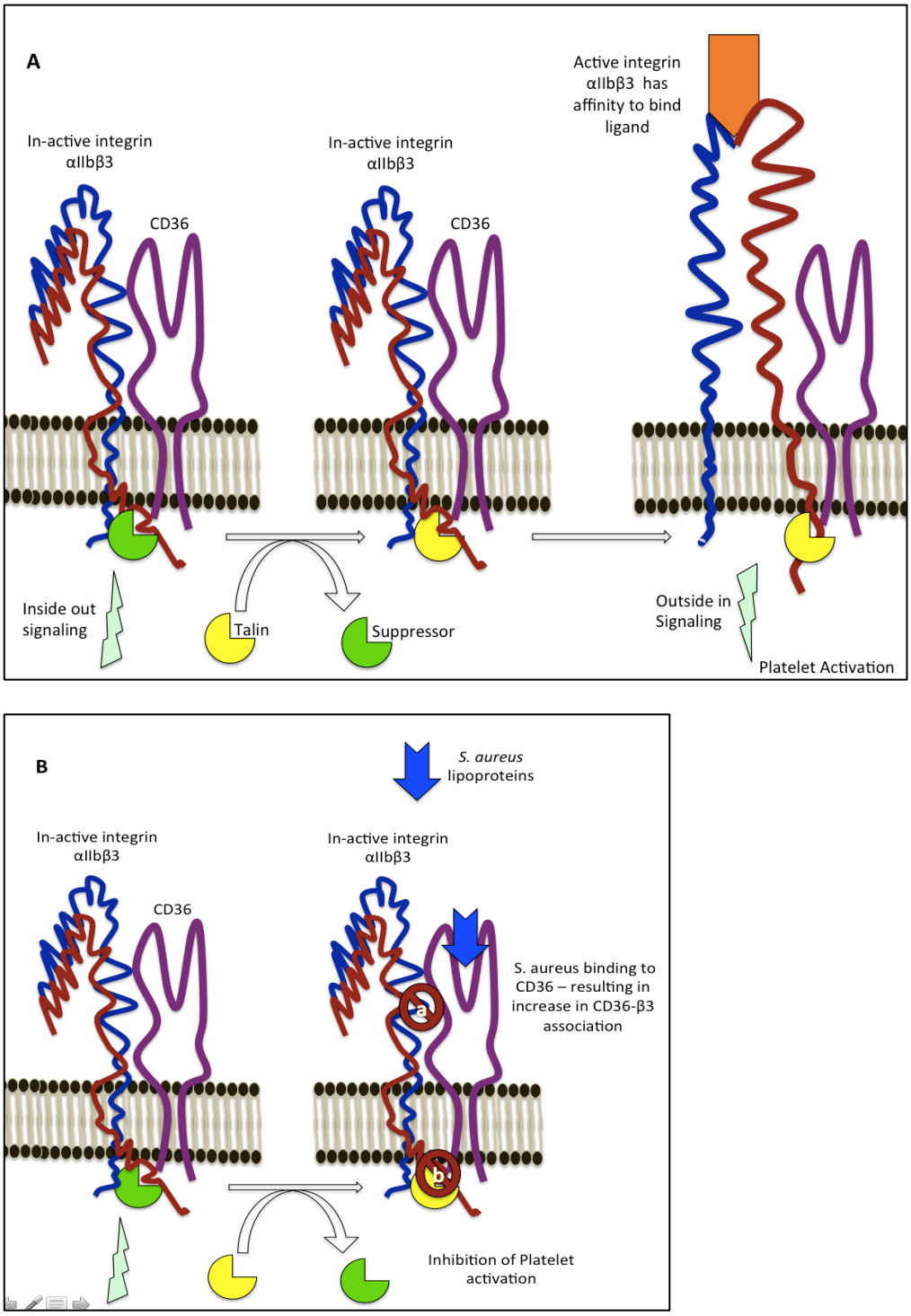

Figure 7.1: Proposed mechanism of inhibition of platelet activation by* S. aureus *lipoproteins via CD36. ***A****. Mechanism of activation of integrin αIIbβ3. Clasping and unclasping of integrin αIIbβ3 is dependent on equilibrium between suppressor or activator (talin) to integrin β3 (Ma et al. 2007).* ***B.*** *Proposed model of blockage of integrin αIIbβ3 conformation change in presence of* S. aureus *lipoproteins. Increase in CD36 - β3 association might* ***a.*** *directly inhibit change in integrin β3 conformation or* ***b.*** *inhibit binding of activator proteins to integrin β3.*

7.4. *S. aureus* lipoproteins inhibit platelet activation – significance in host pathogen interaction.

S. aureus – platelet interaction is speculated to be of significance in pathogenesis of bacteria, especially in bacterial endocarditis (Fitzgerald *et al.* 2006). Initiation of *S. aureus* – platelet plug formation is thought to be occurring via interaction of circulating *S. aureus* with platelets adhered to the damaged vessel surface (Werdan *et al.* 2014). Numerous surface molecules of *S. aureus* bind to platelets and even regulate platelet function but in any interaction (O'Brien *et al.* 2002), avidity of the molecules interacting is significant. In a bacteria platelet interaction, along with the avidity between bacterial surface molecules interacting with platelet surface molecules, their number also plays a significant role. More the bacterial surface molecules able to bind platelets, readily the initiation of infection might occur. Bacteria mediated platelet aggregation lag time is considered to be a marker in understanding the number of surface molecules involved in interaction, lesser the lag time more readily the binding (Fitzgerald *et al.* 2006), thus more surface molecules involved in interaction.

In in-vivo conditions, which is reported to be ion depleted condition for bacteria, *S. aureus* lipoproteins might be expressed in high number as most of them are involved in ion transport (Sheldon & Heinrichs 2012; Horsburgh *et al.* 2002). In an *in vitro* aggregation assay using *S. aureus* cell envelope extracts lacking lipoproteins showed significantly higher lag time compared to *S. aureus* envelope extract with lipoproteins. Even the binding assay showed that *S. aureus* whole bacteria could bind significantly better in presence of lipoproteins. So it

can be speculated that in in-vivo ion limited conditions with high number of lipoproteins on the surface, *S. aureus* lipoproteins might be a significant component in initiation of *S. aureus* – platelet interaction.

The platelet clot from *S. aureus* mediated bacterial endocarditis has reported to show the presence of *S. aureus* inside the plug (Pawar *et al.* 2004). This observation suggests that *S. aureus* survives in this environment and might even proliferate. *S. aureus* has ability to directly bind platelets (O'Brien et al. 2002), has surface molecules that bind plasma proteins (Loughman *et al.* 2005) and can release the molecules that can regulate platelet function (Schubert *et al.* 2011). Thus one can speculate that *S. aureus* mimics the platelets to exist inside the thrombus. In the process of normal thrombus formation platelet release molecules that activate and recruit additional platelets, but it also inhibit platelet activation to control growing thrombus (Vassbotn *et al.* 1994). *S. aureus* also releases the platelet inhibitory molecules (Waller *et al.* 2013; Shannon & Flock 2004) that might play the same role. Along with controlling growing thrombi, ability of *S. aureus* to inhibit platelet activation might contribute in release of bacteria into the blood flow to spread the infection. *S. aureus* lipoproteins are released into the extracellular milieu (Stoll *et al.* 2005). So inside a thrombus, if *S. aureus* grows, along with other platelet inhibitory molecules like LTA and Efb, it will also release its lipoproteins which will contribute in inhibiting platelet activation.

S. aureus lipoproteins also stimulate endothelial cells to release NO (Kim *et al*. 2015), which is a potent inhibitor of platelet activation and also a vasodilator (Gardiner *et al*. 1990). Thus, inhibition of platelet activation and

vasodilation that increases the blood flow, might aid in rapid spread of *S. aureus* into the host. Another advantage of *S. aureus* lipoproteins causing inhibition of platelet activation and NO release for *S. aureus* might be in evasion of immune system. Activated platelets release granules that aid in recruitment of immune cells (von Hundelshausen & Weber 2007). So inhibition of platelet activation and release of NO which increases blood flow and inhibits leukocyte adhesion (Kubes *et al*. 1991) might aid *S. aureus* in avoiding encounter with host immune cells.

7.5. Future work

7.5.1. *S. aureus* lipoproteins involved in binding to platelet CD36.

There are more than 50 putative lipoproteins predicted in *S. aureus* by *in silico* analysis (Sheldon & Heinrichs 2012). The TX-114 extract of *S. aureus* showed the presence of numerous bands on SDS PAGE and co-immunoprecipitation of CD36 with *S. aureus* membrane proteins showed receptor binds multiple proteins. The platelet inhibitory effect of *S. aureus* membrane proteins was only seen in extracts from *S. aureus ΔltaS* but not from *S. aureus lgt ΔltaS* that suggests involvement of *S. aureus* lipoproteins in functional blocking of platelets. Further characterization of these *S. aureus* lipoproteins involved in binding to CD36 and inhibiting platelet activation is necessary.

Separation and isolation of bacterial lipoproteins is difficult due presence of hydrophobic moiety, but use of hydrophobic interaction high performance liquid chromatography (HPLC) is proposed to be successful (Hashimoto *et al.* 2004). Even though it has been seen that gram positive bacteria like *S. aureus* lack Lnt homolog of gram negative bacteria responsible for triacylation of lipoproteins, it is being reported that *S. aureus* MntC lipoprotein can exist in

triacylated form (Asanuma *et al.* 2011). Further evaluation of acylation nature of separated *S. aureus* lipoproteins using technique like Mass spectrometry (Kwok *et al.* 2011) will also be necessary, as acylation nature of lipopeptide might change the platelet response and the receptor involved.

S. aureus lipoproteins are proposed to be a vaccine candidate for *S. aureus* prophylaxis (Anderson *et al*. 2012). Knowledge of the specific lipoproteins and each of their effect on platelet function and on other cell type will provide insights for designing an appropriate vaccine.

7.5.2. Inhibition of platelet activation via CD36.

Binding of ligands to platelet CD36 is known to cause platelet activation (Magwenzi *et al*. 2015), but in the current study it was shown that *S. aureus* lipoproteins inhibit platelet activation via CD36. The interaction between lipoproteins and platelet CD36 did not inhibit initial tyrosine phosphorylation caused by the agonist and it also did not cause any VASP phosphorylation. It did increase the CD36 - β3 association that was checked by co-precipitation assays.

Further evaluation on CD36 - β3 association using high throughput FRET (Förster Resonance Energy Transfer) technology which can evaluate the interaction of surface receptors on the cell surface (Maurel *et al.* 2008) can provide significant information on interaction happening on the cell. Also high resolution microscopy like scanning electron microscopy or super resolution fluorescence microscope (Huang *et al.* 2009) will aid in understanding of changes in surface receptors especially CD36 and β3 happening in presence of *S. aureus* lipoproteins.

Confirmation of changes in association between CD36 and β3 might further led to understanding the mechanism by which the actual inhibition occurs. The current study focused on looking at the association of CD36 with β3, αIIb, CD9 and FcγRIIa in presence of *S. aureus* lipoproteins by using co-precipitation experiments. Change in association between CD36 and β3 was seen but it is being reported that CD36 can associate with the other surface receptors which we looked at (Miao *et al.* 2001), but no association was found, thus it cannot be completely ruled out that *S. aureus* lipoproteins do not affect association of CD36 with other surface proteins on platelets. So using above mentioned techniques the association between CD36 and other surface proteins can be looked at.

Another aspect that should be explored is the effect of *S. aureus* lipoproteins on mouse platelets. The mouse platelets were not affected by the treatment of *S. aureus* lipoproteins. Major platelet surface receptor known to be involved in *S. aureus* mediated human platelet activation is FcγRIIa. Mouse platelets lack FcγRIIa (McKenzie *et al.* 1999) and it should be explored whether its presence on human platelets influences *S. aureus* lipoproteins mediated inhibition. Use of transgenic mice model consisting FcγRIIa receptor (McKenzie *et al.* 1999) for analyzing the effect of *S. aureus* lipoproteins might give insight into the role of this receptor in *S. aureus* lipoproteins mediated inhibition of platelet activation.

7.5.2. Effect of CD36 - β3 association on platelet function.

Platelet integrin αIIbβ3 activation is a complex mechanism involving activation and interaction of numerous proteins (Ma *et al*. 2007). Association of

CD36 with integrin β3 in the platelets in resting state is already known (Miao *et al*. 2001) but its functional significance has not been elucidated. CD36 interacts with other receptors and aids in their function (Erdman *et al*. 2009), but the way in which association of CD36 with integrin β3 affects platelet function is unknown. This study showed that an increase in CD36-β3 association might result in inhibition of platelet activation and this needs to be further explored which can be done by analyzing CD36 knockout mice. Also *S. aureus* lipoproteins can be used as a tool to explore the functional significance of association between CD36 and β3. The further evaluation in this aspect might led to be a novel inhibitory mechanism that can be exploited for designing antithrombotic drug.

References

Abe, T., Shimamura, M., Jackman, K., et al., 2010. Key role of CD36 in Toll-like receptor 2 signaling in cerebral ischemia. *Stroke; a journal of cerebral circulation*, 41(5), pp.898–904.

Aburima, A., Wraith, K.S., Raslan, Z., *et al*., 2013. cAMP signaling regulates platelet myosin light chain (MLC) phosphorylation and shape change through targeting the RhoA-Rho kinase-MLC phosphatase signaling pathway. *Blood*, 122(20), pp.3533–45.

Alderson, B.R., Armitage, R.J., Tough, T.W., *et al.*, 1993. CD40 expression by Human monocytes: Regulation by cytokine and activation of monocytes by the ligand for CD40. *The Journal of Experimental Medicine*, 178(August), pp.669 – 674.

Aliprantis, A.O., Yang, R., Mark, M.R., *et al*., 1999. Cell Activation and Apoptosis by Bacterial Lipoproteins Through Toll-like Receptor-2. *Science*, 285(5428), pp.736–739.

Aly, R., Shinefield, H.R., Litz, C. & Maibach, H.I., 1980. Role of teichoic acid in the binding of *Staphylococcus aureus* to nasal epithelial cells. *The Journal of infectious diseases*, 141(4), pp.463–5.

Andersen, H., Greenberg, D.L., Fujikawa, K., *et al*., 1999. Protease-activated receptor 1 is the primary mediator of thrombin-stimulated platelet procoagulant activity. *Proceedings of the National Academy of Sciences of the United States of America*, 96(20), pp.11189–93.

Anderson, A.S., Miller, A.A., Donald, R.G.K., *et al.*, 2012. Development of a

multicomponent *Staphylococcus aureus* vaccine designed to counter multiple bacterial virulence factors. *Human vaccines & immunotherapeutics*, 8(11), pp.1585–94.

André, P., Prasad, K.S.S., Denis, C. V, *et al*., 2002. CD40L stabilizes arterial thrombi by a beta3 integrin dependent mechanism. *Nature medicine*, 8(3), pp.247–52.

Archer, N.K., Mazaitis, M.J., Costerton, J.W., *et al*., 2011. *Staphylococcus aureus* biofilms: properties, regulation, and roles in human disease. *Virulence*, 2(5), pp.445–59.

Argudín, M.Á., Mendoza, M.C. & Rodicio, M.R., 2010. Food Poisoning and *Staphylococcus aureus* Enterotoxins. *Toxins*, 2(7), pp.1751–1773.

Arman, M., Krauel, K., Tilley, D.O., et al., 2014. Amplification of bacteria-induced platelet activation is triggered by FcγRIIA, integrin αIIbβ3, and platelet factor 4. *Blood*, 123(20), pp.3166–74.

Asanuma, M., Kurokawa, K., Ichikawa, R., *et al*., 2011. Structural evidence of α-aminoacylated lipoproteins of *Staphylococcus aureus*. *The FEBS journal*, 278(5), pp.716–728.

Aslam, R., Speck, E.R., Kim, M., *et al*., 2006. Platelet Toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor-alpha production *in vivo*. *Blood*, 107(2), pp.637–641.

Asselin, J., Gibbins, J.M., Achison, M., *et al*., 1997. A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1. *Blood*, 89(4), pp.1235–42.

Aszodi, A., Pfeifer, A., Ahmad, M., *et al*., 1999. The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *The EMBO Journal*, 18(1), pp.37–48.

Atkins, K.L., Burman, J.D., Chamberlain, E.S., *et al*., 2008. *S. aureus* IgG-binding proteins SpA and Sbi: host specificity and mechanisms of immune complex formation. *Molecular immunology*, 45(6), pp.1600–11.

Badimon, L., Badimon, J.J., Turitto, V.T. & Fuster, V., 1989. Role of von Willebrand factor in mediating platelet-vessel wall interaction at low shear rate; the importance of perfusion conditions. *Blood*, 73(4), pp.961–7.

Baird-Parker, A.C., 1963. A classification of micrococci and staphylococci based on physiological and biochemical tests. *Journal of general microbiology*, 30(3), pp.409–27.

Baldassare, J.J., Henderson, P.A. & Fisher, G.J., 1989. Plasma membrane associated phospholipase C from human platelets: synergistic stimulation of phosphatidylinositol 4,5-bisphosphate hydrolysis by thrombin and guanosine 5'-O-(3-thiotriphosphate). *Biochemistry*, 28(1), pp.56–60.

Banno, Y., Asano, T. & Nozawa, Y., 1998. Stimulation by G protein beta gamma subunits of phospholipase C beta isoforms in human platelets. *Thrombosis and haemostasis*, 79(5), pp.1008–13.

Bantel, H., Sinha, B., Domschke, W., *et al*., 2001. Alpha-Toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling. *The*

Journal of cell biology, 155(4), pp.637–48.

Barbu, E.M., Ganesh, V.K., Gurusiddappa, S., *et al*., 2010. Beta-Neurexin is a ligand for the *Staphylococcus aureus* MSCRAMM SdrC. *PLoS pathogens*, 6(1), p.e1000726.

Barbu, E.M., Mackenzie, C., Foster, T.J. & Höök, M., 2014. SdrC induces staphylococcal biofilm formation through a homophilic interaction. *Molecular microbiology*, 94(1), pp.172–85.

Barrio, B., Vangroenweghe, F., Dosogne, H. & Burvenich, C., 2000. Decreased neutrophil bactericidal activity during phagocytosis of a slime-producing *Staphylococcus aureus* strain. *Veterinary research*, 31(6), pp.603–9.

Baruch, D., Denis, C., Marteaux, C., *et al*., 1991. Role of von Willebrand factor associated to extracellular matrices in platelet adhesion. *Blood*, 77(3), pp.519–27.

Basani, R.B., Zhu, H., Thornton, M.A., *et al*., 2008. Species differences in small molecule binding to alpha IIb beta 3 are the result of sequence differences in 2 loops of the alpha IIb propeller. *Blood*, 113(4), pp.902–910.

Baur, S., Rautenberg, M., Faulstich, M., *et al*., 2014. A nasal epithelial receptor for *Staphylococcus aureus* WTA governs adhesion to epithelial cells and modulates nasal colonization. *PLoS pathogens*, 10(5), p.e1004089.

Bayer, a S., Ramos, M.D., Menzies, B.E., *et al*., 1997. Hyperproduction of alpha-toxin by *Staphylococcus aureus* results in paradoxically reduced virulence in experimental endocarditis: a host defence role for platelet microbicidal

proteins. *Infection and immunity*, 65(11), pp.4652–4660.

Becker, S., Frankel, M.B., Schneewind, O. & Missiakas, D., 2014. Release of protein A from the cell wall of *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the United States of America*, 111(4), pp.1574–9.

Bellamy, T.C., Wood, J., Goodwin, D.A. & Garthwaite, J., 2000. Rapid desensitization of the nitric oxide receptor, soluble guanylyl cyclase, underlies diversity of cellular cGMP responses. *Proceedings of the National Academy of Sciences of the United States of America*, 97(6), pp.2928–33.

Bennett, J.S., 2005. Structure and function of the platelet integrin alphaIIbbeta3. *The Journal of clinical investigation*, 115(12), pp.3363–9.

Berger, G., Massé, J.M. & Cramer, E.M., 1996. Alpha-granule membrane mirrors the platelet plasma membrane and contains the glycoproteins Ib, IX, and V. *Blood*, 87(4), pp.1385–95.

Bergmeier, W. & Hynes, R.O., 2012. Extracellular matrix proteins in hemostasis and thrombosis. *Cold Spring Harbor perspectives in biology*, 4(2), p.a005132–.

Berman, C.L., Yeo, E.L., Wencel-Drake, J.D., *et al*., 1986. A platelet alpha granule membrane protein that is associated with the plasma membrane after activation. Characterization and subcellular localization of platelet activation-dependent granule-external membrane protein. *The Journal of clinical investigation*, 78(1), pp.130–7.

Bessler, W.G., Cox, M., Lex, A., *et al*., 1985. Synthetic lipopeptide analogs of

bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. *Journal of immunology (Baltimore, Md. : 1950)*, 135(3), pp.1900–1905.

Bhakdi, S. & Tranum-Jensen, J., 1991. Alpha-toxin of *Staphylococcus aureus*. *Microbiological reviews*, 55(4), pp.733–51.

Bhatia, a & Zahoor, S., 2007. *Staphylococcus aureus* enterotoxins: A review. *Journal of Clinical Diagnoses Research*, 1(3), pp.188–197.

Blair, P. & Flaumenhaft, R., 2009. Platel*et al*pha-granules: basic biology and clinical correlates. *Blood reviews*, 23(4), pp.177–89.

Blair, P., Rex, S., Vitseva, O., *et al*., 2009. Stimulation of Toll-like receptor 2 in human platelets induces a thromboinflammatory response through activation of phosphoinositide 3-kinase. *Circulation research*, 104(3), pp.346–354.

Blanc, L., Castanier, R., Mishra, A.K., *et al*., 2013. Gram-positive bacterial lipoglycans based on a glycosylated diacylglycerol lipid anchor are microbe-associated molecular patterns recognized by TLR2. *PloS one*, 8(11), p.e81593.

Borsutzky, S., Kretschmer, K., Becker, P.D., *et al*., 2005. The Mucosal Adjuvant Macrophage-Activating Lipopeptide-2 Directly Stimulates B Lymphocytes via the TLR2 without the Need of Accessory Cells. *The Journal of Immunology*, 174(10), pp.6308–6313.

Boulaftali, Y., Hess, P.R., Kahn, M.L. & Bergmeier, W., 2014. Platelet

immunoreceptor tyrosine-based activation motif (ITAM) signaling and vascular integrity. *Circulation research*, 114(7), pp.1174–84.

Brauweiler, A.M., Goleva, E. & Leung, D.Y.M., 2014. Th2 cytokines increase *Staphylococcus aureus* alpha toxin-induced keratinocyte death through the signal transducer and activator of transcription 6 (STAT6). *The Journal of investigative dermatology*, 134(8), pp.2114–21.

Brightbill, H.D., Libraty, D.H., Krutzik, S.R., *et al*., 1999. Host defence mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science (New York, N.Y.)*, 285(5428), pp.732–736.

Bubeck Wardenburg, J., Patel, R.J. & Schneewind, O., 2007. Surface proteins and exotoxins are required for the pathogenesis of *Staphylococcus aureus* pneumonia. *Infection and immunity*, 75(2), pp.1040–4.

Bubeck Wardenburg, J., Williams, W.A. & Missiakas, D., 2006. Host defences against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins. *Proceedings of the National Academy of Sciences of the United States of America*, 103(37), pp.13831–6.

Bukowski, M., Wladyka, B. & Dubin, G., 2010. Exfoliative toxins of *Staphylococcus aureus*. *Toxins*, 2(5), pp.1148–65.

Bull, H.A., Brickell, P.M. & Dowd, P.M., 1994. Src-related protein tyrosine kinases are physically associated with the surface antigen CD36 in human dermal microvascular endothelial cells. *FEBS letters*, 351(1), pp.41–4.

Buwitt-Beckmann, U., Heine, H., Wiesmüller, K.-H., *et al*., 2005. Toll-like receptor

6-independent signaling by diacylated lipopeptides. *European journal of immunology*, 35(1), pp.282–9.

Canobbio, I., Balduini, C. & Torti, M., 2004. Signalling through the platelet glycoprotein Ib-V-IX complex. *Cellular signalling*, 16(12), pp.1329–44.

Canton, J., Neculai, D. & Grinstein, S., 2013. Scavenger receptors in homeostasis and immunity. *Nature reviews. Immunology*, 13(9), pp.621–34.

Cerletti, C., De Gaetano, G. & Lorenzet, R., 2010. Platelet - leukocyte interactions: multiple links between inflammation, blood coagulation and vascular risk. *Mediterranean journal of hematology and infectious diseases*, 2(3).

Chatterjee, D. & Anderson, G.M., 1993. The human platelet dense granule: serotonin uptake, tetrabenazine binding, phospholipid and ganglioside profiles. *Archives of biochemistry and biophysics*, 302(2), pp.439–46.

Chen, W.-F., Lee, J.-J., Chang, C.-C., *et al*., 2013. Platelet protease-activated receptor (PAR)4, but not PAR1, associated with neutral sphingomyelinase responsible for thrombin-stimulated ceramide-NF-κB signaling in human platelets. *Haematologica*, 98(5), pp.793–801.

Chiller, K., Selkin, B.A. & Murakawa, G.J., 2001. Skin microflora and bacterial infections of the skin. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research*, 6(3), pp.170–4.

Ciferri, S., Emiliani, C., Guglielmini, G., *et al*., 2000. Platelets release their lysosomal content in vivo in humans upon activation. *Thrombosis and*

haemostasis, 83(1), pp.157–64.

Clarke, S.R., Andre, G., Walsh, E.J., *et al*., 2009. Iron-regulated surface determinant protein A mediates adhesion of *Staphylococcus aureus* to human corneocyte envelope proteins. *Infection and immunity*, 77(6), pp.2408–16.

Clarke, S.R. & Foster, S.J., 2006. Surface adhesins of *Staphylococcus aureus*. *Advances in microbial physiology*, 51, pp.187–224.

Clarke, S.R., Harris, L.G., Richards, R.G. & Foster, S.J., 2002. Analysis of Ebh, a 1.1-megadalton cell wall-associated fibronectin-binding protein of *Staphylococcus aureus*. *Infection and immunity*, 70(12), pp.6680–7.

Clarke, S.R., Wiltshire, M.D. & Foster, S.J., 2004. IsdA of *Staphylococcus aureus* is a broad spectrum, iron-regulated adhesin. *Molecular microbiology*, 51(5), pp.1509–19.

Clauditz, A., Resch, A., Wieland, K.-P., *et al*., 2006. Staphyloxanthin plays a role in the fitness of *Staphylococcus aureus* and its ability to cope with oxidative stress. *Infection and immunity*, 74(8), pp.4950–3.

Cockayne, A., Hill, P.J., Powell, N.B., *et al*., 1998. Molecular cloning of a 32-kilodalton lipoprotein component of a novel iron-regulated Staphylococcus epidermidis ABC transporter. *Infection and immunity*, 66(8), pp.3767–3774.

Cognasse, F., Hamzeh, H., Chavarin, P., *et al*., 2005. Evidence of Toll-like receptor molecules on human platelets. *Immunol Cell Biol*, 83(2), pp.196–198.

Coley, J., Archibald, A.R. & Baddiley, J., 1976. A linkage unit joining peptidoglycan to teichoic acid in *Staphylococcus aureus* H. *FEBS Letters*, 61(2), pp.240–242.

Corrigan, R.M., Abbott, J.C., Burhenne, H., *et al*., 2011. c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. *PLoS pathogens*, 7(9), p.e1002217.

Cox, D., Kerrigan, S.W. & Watson, S.P., 2011. Platelets and the innate immune system: mechanisms of bacterial-induced platelet activation. *Journal of thrombosis and haemostasis : JTH*, 9(6), pp.1097–107.

Cramton, S.E., Ulrich, M., Götz, F. & Döring, G., 2001. Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and Staphylococcus epidermidis. *Infection and immunity*, 69(6), pp.4079–85.

Crane, M.S., Rossi, A.G. & Megson, I.L., 2005. A potential role for extracellular nitric oxide generation in cGMP-independent inhibition of human platelet aggregation: biochemical and pharmacological considerations. *British journal of pharmacology*, 144(6), pp.849–59.

Crook, M., 1991. Sialic Acid: its importance to platelet function in health and disease. *Platelets*, 2(1), pp.1–10.

Cserti-Gazdewich, C.M., Dzik, W.H., Dorn, M.E., *et al*., 2009. Quantitation of CD36 (platelet glycoprotein IV) expression on platelets and monocytes by flow cytometry: application to the study of Plasmodium falciparum malaria. *Cytometry. Part B, Clinical cytometry*, 76(2), pp.127–34.

Daniel, J.L., Dangelmaier, C., Jin, J., *et al*., 1999. Role of intracellular signaling events in ADP-induced platelet aggregation. *Thrombosis and haemostasis*, 82(4), pp.1322–6.

Defawe, O.D., Kim, S., Chen, L., *et al*., 2010. VASP phosphorylation at serine239 regulates the effects of NO on smooth muscle cell invasion and contraction of collagen. *Journal of cellular physiology*, 222(1), pp.230–7.

Deiters, U. & Mühlradt, P.F., 1999. Mycoplasmal lipopeptide MALP-2 induces the chemoattractant proteins macrophage inflammatory protein 1alpha (MIP-1alpha), monocyte chemoattractant protein 1, and MIP-2 and promotes leukocyte infiltration in mice. *Infection and immunity*, 67(7), pp.3390–8.

Dell'Angelica, E.C., 2000. Lysosome-related organelles. *The FASEB Journal*, 14(10), pp.1265–1278.

Deutsch, V.R. & Tomer, A., 2006. Megakaryocyte development and platelet production. *British journal of haematology*, 134(5), pp.453–66.

Diaz-Ricart, M., Etebanell, E., Cases, A., *et al*., 1999. Erythropoietin improves signaling through tyrosine phosphorylation in platelets from uremic patients. *Thrombosis and haemostasis*, 82(4), pp.1312–7.

Dickfeld, T., Lengyel, E., May, E., *et al*., 2001. Transient interaction of activated platelets with endothelial cells induces expression of monocyte-chemoattractant protein-1 via a p38 mitogen-activated protein kinase mediated pathway. Implications for atherogenesis. *Cardiovascular research*, 49(1), pp.189–99.

Donlan, R.M. & Costerton, J.W., 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical microbiology reviews*, 15(2), pp.167–93.

Dorrington, K.J., 1976. Properties of the Fc receptor on macrophages.

Immunological communications, 5(4), pp.263–80.

DuMont, A.L., Yoong, P., Surewaard, B.G.J., *et al*., 2013. *Staphylococcus aureus* elaborates leukocidin AB to mediate escape from within human neutrophils. *Infection and immunity*, 81(5), pp.1830–41.

Edwards, A.M., Potts, J.R., Josefsson, E. & Massey, R.C., 2010. *Staphylococcus aureus* Host Cell Invasion and Virulence in Sepsis Is Facilitated by the Multiple Repeats within FnBPA. A. Cheung, ed. *PLoS Pathogens*, 6(6), p.e1000964.

Emsley, J., Knight, C.G., Farndale, R.W., *et al*., 2000. Structural basis of collagen recognition by integrin alpha2beta1. *Cell*, 101(1), pp.47–56.

Erdman, L.K., Cosio, G., Helmers, A.J., *et al*., 2009. CD36 and TLR interactions in inflammation and phagocytosis: implications for malaria. *Journal of immunology (Baltimore, Md. : 1950)*, 183(10), pp.6452–9.

Ertel, K., Al-Tawil, M., Santoso, S. & Kroll, H., 2005. Relevance of the HPA-15 (Gov) polymorphism on CD109 in alloimmune thrombocytopenic syndromes. *Transfusion*, 45(3), pp.366–73.

Escolar, G. & White, J.G., 1991. The platelet open canalicular system: a final common pathway. *Blood cells*, 17(3), pp.467–85; 486–95.

Etulain, J., Martinod, K., Wong, S.L., *et al*., 2015. P-selectin promotes neutrophil extracellular trap formation in mice. *Blood*, 126(2), pp.242–6.

Ezumi, Y., Shindoh, K., Tsuji, M. & Takayama, H., 1998. Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor

glycoprotein VI-Fc receptor gamma chain complex on human platelets. *The Journal of experimental medicine*, 188(2), pp.267–76.

Fälker, K., Klarström-Engström, K., Bengtsson, T., *et al*., 2014. The Toll-like receptor 2/1 (TLR2/1) complex initiates human platelet activation via the src/Syk/LAT/PLCγ2 signalling cascade. *Cellular signalling*, 26(2), pp.279–86.

Finlay, B.B. & Falkow, S., 1997. Common themes in microbial pathogenicity revisited. *Microbiology and molecular biology reviews : MMBR*, 61(2), pp.136–169.

Fitzgerald, J.R., Foster, T.J. & Cox, D., 2006. The interaction of bacterial pathogens with platelets. *Nature reviews. Microbiology*, 4(6), pp.445–457.

Fitzgerald, J.R., Loughman, A., Keane, F., *et al*., 2006. Fibronectin-binding proteins of *Staphylococcus aureus* mediate activation of human platelets via fibrinogen and fibronectin bridges to integrin GPIIb/IIIa and IgG binding to the FcgammaRIIa receptor. *Molecular microbiology*, 59(1), pp.212–230.

Flaumenhaft, R., 2003. Molecular Basis of Platelet Granule Secretion. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 23(7), pp.1152–1160.

Fogelson, A.L. & Wang, N.T., 1996. Platelet dense-granule centralization and the persistence of ADP secretion. *The American journal of physiology*, 270(3 Pt 2), pp.H1131–40.

Foster, T.J., Geoghegan, J., Ganesh, V.K. & Höök, M., 2014. Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus*

aureus. *Nature Reviews Microbiology*, 12(1), pp.49–62.

Foster, T.J. & Höök, M., 1998. Surface protein adhesins of *Staphylococcus aureus*. *Trends in microbiology*, 6(12), pp.484–488.

Frenette, P.S., Johnson, R.C., Hynes, R.O. & Wagner, D.D., 1995. Platelets roll on stimulated endothelium in vivo: an interaction mediated by endothelial P-selectin. *Proceedings of the National Academy of Sciences of the United States of America*, 92(16), pp.7450–4.

Friebe, A. & Koesling, D., 2003. Regulation of nitric oxide-sensitive guanylyl cyclase. *Circulation research*, 93(2), pp.96–105.

Furie, B., Furie, B.C. & Flaumenhaft, R., 2001. A journey with platelet P-selectin: The molecular basis of granule secretion, signalling and cell adhesion. *Thrombosis and Haemostasis*, 86(1), pp.214–221.

Falati, S., Edmead, C.E. & Poole, A.W., 1999. Glycoprotein Ib-V-IX, a receptor for von Willebrand factor, couples physically and functionally to the Fc receptor gamma-chain, Fyn, and Lyn to activate human platelets. *Blood*, 94(5), pp.1648–56.

Galanos, C., Gumenscheimer, M., Mühlradt, P., *et al*., 2000. MALP-2, a Mycoplasma lipopeptide with classical endotoxic properties: end of an era of LPS monopoly? *Journal of endotoxin research*, 6(6), pp.471–476.

Ganesh, V.K., Rivera, J.J., Smeds, E., *et al*., 2008. A structural model of the *Staphylococcus aureus* ClfA-fibrinogen interaction opens new avenues for the design of anti-staphylococcal therapeutics. *PLoS pathogens*, 4(11),

p.e1000226.

Gao, J., Zoller, K.E., Ginsberg, M.H., *et al*., 1997. Regulation of the pp72syk protein tyrosine kinase by platelet integrin alpha IIb beta 3. *The EMBO journal*, 16(21), pp.6414–25.

Gardiner, E.E., Arthur, J.F., Shen, Y., *et al*., 2010. GPIbalpha-selective activation of platelets induces platelet signaling events comparable to GPVI activation events. *Platelets*, 21(4), pp.244–52.

Gardiner, S.M., Compton, A.M., Bennett, T., *et al*., 1990. Control of regional blood flow by endothelium-derived nitric oxide. *Hypertension*, 15(5), pp.486–492.

Gaul, V., Lopez, S.G., Lentz, B.R., *et al*., 2015. The lateral diffusion and fibrinogen induced clustering of platelet integrin αIIbβ3 reconstituted into physiologically mimetic GUVs. *Integrative biology : quantitative biosciences from nano to macro*, 7(4), pp.402–11.

Gautam, S., Kim, T., Lester, E., *et al*., 2015. Wall teichoic acids prevent antibody binding to epitopes within the cell wall of *Staphylococcus aureus*. *ACS chemical biology*.

Geiger, J., Nolte, C., Butt, E., *et al*., 1992. Role of cGMP and cGMP dependent protein kinase in nitrovasodilator inhibition of agonist evoked calcium elevation in human platelets. *Proc. Natl. Acad.Sci.*, 89(February), pp.1031–1035.

Georgoutsou-Spyridonos, M., Ricklin, D., Pratsinis, H., *et al*., 2015. Attenuation of *Staphylococcus aureus*-Induced Bacteremia by Human Mini-Antibodies

Targeting the Complement Inhibitory Protein Efb. *Journal of immunology (Baltimore, Md. : 1950)*, 195(8), pp.3946–58.

Ghosh, A., Murugesan, G., Chen, K., *et al*., 2011. Platelet CD36 surface expression levels affect functional responses to oxidized LDL and are associated with inheritance of specific genetic polymorphisms. *Blood*, 117(23), pp.6355–66.

Gkaliagkousi, E., Ritter, J. & Ferro, A., 2007. Platelet-derived nitric oxide signaling and regulation. *Circulation research*, 101(7), pp.654–62.

Golden, A., Nemeth, S.P. & Brugge, J.S., 1986. Blood platelets express high levels of the pp60c-src-specific tyrosine kinase activity. *Proceedings of the National Academy of Sciences of the United States of America*, 83(4), pp.852–6.

Gordon, J.J., Harter, D.H. & Phair, J.P., 1985. Meningitis due to *Staphylococcus aureus*. *The American Journal of Medicine*, 78(6), pp.965–970.

Graille, M., Stura, E.A., Corper, A.L., *et al*., 2000. Crystal structure of a *Staphylococcus aureus* protein A domain complexed with the Fab fragment of a human IgM antibody : Structural basis for recognition of B-cell receptors and superantigen activity. *Proceedings of the National Academy of Sciences of the United States of America*, 97(10), pp.5399–5404.

Gresham, H.D., Lowrance, J.H., Caver, T.E., *et al*., 2000. Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. *Journal of immunology*, 164(7), pp.3713–22.

Gross, M., Cramton, S.E., Götz, F. & Peschel, A., 2001. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infection*

and immunity, 69(5), pp.3423–6.

Grozovsky, R., Begonja, A.J., Liu, K., *et al*., 2014. The Ashwell-Morell receptor regulates hepatic thrombopoietin production via JAK2-STAT3 signaling. *Nature Medicine*, 21(1), pp.47–54.

Gruarin, P., Sitia, R. & Alessio, M., 1997. Formation of one or more intrachain disulphide bonds is required for the intracellular processing and transport of CD36. *The Biochemical journal*, 328 (Pt 2, pp.635–42.

Grüner, S., Prostredna, M., Schulte, V., *et al*., 2003. Multiple integrin-ligand interactions synergize in shear-resistant platelet adhesion at sites of arterial injury in vivo. *Blood*, 102(12), pp.4021–4027.

Gryglewski, R.J., Dembínska-Kieć, A. & Korbut, R., 1978. A possible role of thromboxane A2 (TXA2) and prostacyclin (PGI2) in circulation. *Acta biologica et medica Germanica*, 37(5-6), pp.715–23.

Hair, P.S., Ward, M.D., Semmes, O.J., *et al*., 2008. *Staphylococcus aureus* clumping factor A binds to complement regulator factor I and increases factor I cleavage of C3b. *The Journal of infectious diseases*, 198(1), pp.125–33.

Hamzeh-Cognasse, H., Damien, P., Chabert, A., *et al*., 2015. Platelets and infections - complex interactions with bacteria. *Frontiers in immunology*, 6, p.82.

Hara, T., Shimizu, K., Ogawa, F., *et al*., 2010. Platelets control leukocyte recruitment in a murine model of cutaneous arthus reaction. *The American journal of pathology*, 176(1), pp.259–69.

Harper, M.T. & Poole, A.W., 2010. Diverse functions of protein kinase C isoforms

in platelet activation and thrombus formation. *Journal of thrombosis and haemostasis : JTH*, 8(3), pp.454–62.

Harris, D.G., Benipal, P.K., Cheng, X., *et al*., 2015. Four-dimensional characterization of thrombosis in a live-cell, shear-flow assay: development and application to xenotransplantation. *PloS one*, 10(4), p.e0123015.

Harrison, P. & Cramer, E.M., 1993. Platelet alpha-granules. *Blood reviews*, 7(1), pp.52–62.

Hartford, O., O'Brien, L., Schofield, K., *et al*., 2001. The Fbe (SdrG) protein of *Staphylococcus epidermidis* HB promotes bacterial adherence to fibrinogen. *Microbiology*, 147(Pt 9), pp.2545–2552.

Hartleib, J., Köhler, N., Dickinson, R.B., *et al*., 2000. Protein A is the von Willebrand factor binding protein on *Staphylococcus aureus*. *Blood*, 96(6), pp.2149–2156.

Hartwig, J.H., 2006. The platelet: form and function. *Seminars in hematology*, 43(1 Suppl 1), pp.S94–100.

Hartwig, J.H. & DeSisto, M., 1991. The cytoskeleton of the resting human blood platelet: structure of the membrane skeleton and its attachment to actin filaments. *The Journal of cell biology*, 112(3), pp.407–425.

Hawiger, J., Steckley, S., Hammond, D., *et al*., 1979. Staphylococci-induced human platelet injury mediated by protein A and immunoglobulin G Fc fragment receptor. *The Journal of clinical investigation*, 64(4), pp.931–937.

Heemskerk, J.W.M., Bevers, E.M. & Lindhout, T., 2002. Platelet activation and

blood coagulation. *Thrombosis and haemostasis*, 88(2), pp.186–93.

Heikkinen, O., Seppala, R., Tossavainen, H., *et al*., 2009. Solution structure of the parvulin-type PPIase domain of *Staphylococcus aureus* PrsA--implications for the catalytic mechanism of parvulins. *BMC structural biology*, 9, p.17.

Hertz, C.J., Kiertscher, S.M., Godowski, P.J., *et al*., 2001. Microbial lipopeptides stimulate dendritic cell maturation via Toll-like receptor 2. *Journal of immunology (Baltimore, Md. : 1950)*, 166(4), pp.2444–2450.

Hoebe, K., Georgel, P., Rutschmann, S., et al., 2005. CD36 is a sensor of diacylglycerides. *Nature*, 433(7025), pp.523–7.

Hoosdally, S.J., Andress, E.J., Wooding, C., *et al*., 2009. The Human Scavenger Receptor CD36: glycosylation status and its role in trafficking and function. *The Journal of biological chemistry*, 284(24), pp.16277–88.

Horii, K., Kahn, M.L. & Herr, A.B., 2006. Structural basis for platelet collagen responses by the immune-type receptor glycoprotein VI. *Blood*, 108(3), pp.936–942.

Horsburgh, M.J., Clements, M.O., Crossley, H., *et al*., 2001. PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. *Infection and immunity*, 69(6), pp.3744–54.

Horst, S.A., Hoerr, V., Beineke, A., *et al*., 2012. A Novel Mouse Model of *Staphylococcus aureus* Chronic Osteomyelitis That Closely Mimics the Human Infection. *The American Journal of Pathology*, 181(4), pp.1206–1214.

Hoylaerts, M.F., Yamamoto, H., Nuyts, K., *et al*., 1997. von Willebrand factor binds

to native collagen VI primarily via its A1 domain. *The Biochemical journal*, 324 (Pt 1, pp.185–91.

Hu, D.-L., Zhu, G., Mori, F., *et al*., 2007. Staphylococcal enterotoxin induces emesis through increasing serotonin release in intestine and it is downregulated by cannabinoid receptor 1. *Cellular microbiology*, 9(9), pp.2267–77.

Hugo, F., Klapettek, K. & Mueller, C., 1988. Staphylococcal alpha toxin promotes blood coagulation via attack on human platelets. *The Journal of Experimental Medicine*, 168(August), pp.527–542.

Von Hundelshausen, P., Koenen, R.R., Sack, M., *et al*., 2005. Heterophilic interactions of platelet factor 4 and RANTES promote monocyte arrest on endothelium. *Blood*, 105(3), pp.924–30.

Von Hundelshausen, P. & Weber, C., 2007. Platelets as immune cells: bridging inflammation and cardiovascular disease. *Circulation research*, 100(1), pp.27–40.

Von Hundelshausen, P., Weber, K.S.C., Huo, Y., *et al*., 2001. RANTES Deposition by Platelets Triggers Monocyte Arrest on Inflamed and Atherosclerotic Endothelium. *Circulation*, 103(13), pp.1772–1777.

Hutchings, M.I., Palmer, T., Harrington, D.J. & Sutcliffe, I.C., 2009. Lipoprotein biogenesis in Gram-positive bacteria: knowing when to hold ’em, knowing when to fold 'em. *Trends in microbiology*, 17(1), pp.13–21.

Hwang, S.M., Kim, M.J., Chang, H.E., *et al*., 2013. Human platelet antigen genotyping and expression of CD109 (human platelet antigen 15) mRNA in

various human cell types. *BioMed research international*, 2013, p.946403.

Ikeda, Y., Handa, M., Kawano, K., *et al*., 1991. The role of von Willebrand factor and fibrinogen in platelet aggregation under varying shear stress. *The Journal of clinical investigation*, 87(4), pp.1234–40.

Isenberg, J.S., Romeo, M.J., Yu, C., *et al*., 2008. Thrombospondin-1 stimulates platelet aggregation by blocking the antithrombotic activity of nitric oxide/cGMP signaling. *Blood*, 111(2), pp.613–23.

Israels, S.J., McMillan, E.M., Robertson, C., *et al*., 1996. The lysosomal granule membrane protein, LAMP-2, is also present in platelet dense granule membranes. *Thrombosis and haemostasis*, 75(4), pp.623–9.

Jackson, C.W., 1990. Megakaryocyte endomitosis: a review. *International journal of cell cloning*, 8(4), pp.224–6.

Janabi, M., Yamashita, S., Hirano, K., et al., 2000. Oxidized LDL-induced NF-kappa B activation and subsequent expression of proinflammatory genes are defective in monocyte-derived macrophages from CD36-deficient patients. *Arteriosclerosis, thrombosis, and vascular biology*, 20(8), pp.1953–1960.

Jarvis, G.E., Atkinson, B.T., Snell, D.C. & Watson, S.P., 2002. Distinct roles of GPVI and integrin alpha(2)beta(1) in platelet shape change and aggregation induced by different collagens. *British journal of pharmacology*, 137(1), pp.107–17.

Jenkins, A.L., Nannizzi-Alaimo, L., Silver, D., *et al*., 1998. Tyrosine Phosphorylation of the 3 Cytoplasmic Domain Mediates Integrin-

Cytoskeletal Interactions. *Journal of Biological Chemistry*, 273(22), pp.13878–13885.

Jensen, L.J., Kuhn, M., Stark, M., *et al*., 2009. STRING 8 - a global view on proteins and their functional interactions in 630 organisms. *Nucleic acids research*, 37(Database issue), pp.D412–6.

Jiang, P., Loyau, S., Tchitchinadze, M., *et al*., 2015. Inhibition of Glycoprotein VI Clustering by Collagen as a Mechanism of Inhibiting Collagen-Induced Platelet Responses: The Example of Losartan C. Schulz, ed. *PLOS ONE*, 10(6), p.e0128744.

Jirouskova, M., Shet, A.S. & Johnson, G.J., 2007. A guide to murine platelet structure, function, assays, and genetic alterations. *Journal of thrombosis and haemostasis : JTH*, 5(4), pp.661–9.

Johnson, G.M., Lee, D.A., Regelmann, W.E., *et al*., 1986. Interference with granulocyte function by *Staphylococcus epidermidis* slime. *Infect. Immun.*, 54(1), pp.13–20.

Johnson, W.M., Tyler, S.D., Ewan, E.P., *et al*., 1991. Detection of genes for enterotoxins, exfoliative toxins, and toxic shock syndrome toxin 1 in *Staphylococcus aureus* by the polymerase chain reaction. *J. Clin. Microbiol.*, 29(3), pp.426–430.

Josefsson, E., Mccrea, K.W., Eidhin, D.N., *et al*., 1998. Three new members of the serine-aspartate repeat protein multigene family of *Staphylococcus aureus*. *Microbiology*, 144, pp.3387–3395.

Jung, S.M., Moroi, M., Soejima, K., *et al*., 2012. Constitutive dimerization of glycoprotein VI (GPVI) in resting platelets is essential for binding to collagen and activation in flowing blood. *The Journal of biological chemistry*, 287(35), pp.30000–13.

Jurk, K., Clemetson, K.J., De Groot, P.G., *et al*., 2003. Thrombospondin-1 mediates platelet adhesion at high shear via glycoprotein Ib (GPIb): an alternative/backup mechanism to von Willebrand factor. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 17(11), pp.1490–2.

Kahn, M.L., Nakanishi-Matsui, M., Shapiro, M.J., *et al*., 1999. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *The Journal of clinical investigation*, 103(6), pp.879–87.

Kahn, M.L., Zheng, Y.W., Huang, W., *et al*., 1998. A dual thrombin receptor system for platelet activation. *Nature*, 394(6694), pp.690–4.

Kahner, B.N., Kato, H., Banno, A., *et al*., 2012. Kindlins, integrin activation and the regulation of talin recruitment to αIIbβ3. *PloS one*, 7(3), p.e34056.

Kälvegren, H., Skoglund, C., Helldahl, C., *et al*., 2010. Toll-like receptor 2 stimulation of platelets is mediated by purinergic P2X1-dependent Ca^{2+} mobilisation, cyclooxygenase and purinergic P2Y1 and P2Y12 receptor activation. *Thrombosis and haemostasis*, 103(2), pp.398–407.

Kang, S.-S., Noh, S.Y., Park, O.-J., *et al*., 2015. *Staphylococcus aureus* induces IL-8 expression through its lipoproteins in the human intestinal epithelial cell, Caco-2. *Cytokine*, 75(1), pp.174–80.

Kaplan, J.E. & Saba, T.M., 1978. Platelet removal from the circulation by the liver and spleen. *Am J Physiol Heart Circ Physiol*, 235(3), pp.H314–320.

Kashiwagi, H., Schwartz, M.A., Eigenthaler, M., *et al*., 1997. Affinity modulation of platelet integrin alphaIIbbeta3 by beta3-endonexin, a selective binding partner of the beta3 integrin cytoplasmic tail. *The Journal of cell biology*, 137(6), pp.1433–43.

Keane, F.M., Loughman, A., Valtulina, V., *et al*., 2007. Fibrinogen and elastin bind to the same region within the A domain of fibronectin binding protein A, an MSCRAMM of *Staphylococcus aureus*. *Molecular microbiology*, 63(3), pp.711–23.

Kerrigan, S.W., Clarke, N., Loughman, A., et al., 2008. Molecular basis for Staphylococcus aureus-mediated platelet aggregate formation under arterial shear in vitro. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 28(2), pp.335–340.

Kim, C., Lau, T.-L., Ulmer, T.S. & Ginsberg, M.H., 2009. Interactions of platelet integrin alphaIIb and beta3 transmembrane domains in mammalian cell membranes and their role in integrin activation. *Blood*, 113(19), pp.4747–53.

Kim, K.W., Im, J., Jeon, J.H., *et al*., 2011. *Staphylococcus aureus* induces IL-1β expression through the activation of MAP kinases and AP-1, CRE and NF-κB transcription factors in the bovine mammary gland epithelial cells. *Comparative immunology, microbiology and infectious diseases*, 34(4), pp.347–54.

Kim, N.J., Ahn, K.B., Jeon, J.H., *et al*., 2015. Lipoprotein in the cell wall of *Staphylococcus aureus* is a major inducer of nitric oxide production in murine macrophages. *Molecular immunology*, 65(1), pp.17–24.

Kintarak, S., Whawell, S.A., Speight, P.M., *et al*., 2004. Internalization of *Staphylococcus aureus* by human keratinocytes. *Infection and immunity*, 72(10), pp.5668–75.

Kita, A., Sakurai, Y., Myers, D.R., *et al*., 2011. Microenvironmental geometry guides platelet adhesion and spreading: a quantitative analysis at the single cell level. *PloS one*, 6(10), p.e26437.

Klockenbusch, W., Hohlfeld, T., Wilhelm, M., *et al*., 1996. Platelet PGI2 receptor affinity is reduced in pre-eclampsia. *British journal of clinical pharmacology*, 41(6), pp.616–8.

Knezevic, I., Leisner, T.M. & Lam, S.C.-T., 1996. Direct Binding of the Platelet Integrin GPIIb-IIIa to Talin: Evidence that interaction is mediated through the cytoplasmic domains of both alpha IIb and beta 3. *Journal of Biological Chemistry*, 271(27), pp.16416–16421.

Kobayashi, S.D. & DeLeo, F.R., 2013. *Staphylococcus aureus* protein A promotes immune suppression. *mBio*, 4(5), pp.e00764–13.

Koedam, J.A., Cramer, E.M., Briend, E., *et al*., 1992. P-selectin, a granule membrane protein of platelets and endothelial cells, follows the regulated secretory pathway in AtT-20 cells. *The Journal of cell biology*, 116(3), pp.617–25.

Kohler, T., Weidenmaier, C. & Peschel, A., 2009. Wall teichoic acid protects *Staphylococcus aureus* against antimicrobial fatty acids from human skin. *Journal of bacteriology*, 191(13), pp.4482–4.

Konopatskaya, O., Matthews, S.A., Harper, M.T., *et al*., 2011. Protein kinase C mediates platelet secretion and thrombus formation through protein kinase D2. *Blood*, 118(2), pp.416–24.

Kovacs-Simon, A., Titball, R.W. & Michell, S.L., 2011. Lipoproteins of bacterial pathogens. *Infection and immunity*, 79(2), pp.548–561.

Kralisz, U. & Cierniewski, C.S., 2000. Activity of pp60c-src and association of pp60c-src, pp54/58lyn, pp60fyn, and pp72syk with the cytoskeleton in platelets activated by collagen. *IUBMB life*, 49(1), pp.33–42.

Krauel, K., Tilley, D.O., Weber, C., et al., 2014. Amplification of bacteria-induced platelet activation is triggered by FcgRIIA , integrin aIIbb3 , and platelet factor 4. *Blood*, 123(20), pp.3166–3175.

Krasilnikov, O. V, Sabirov, R.Z., Ternovsky, V.I. & Tashmukhamedov, B.A., 1988. The Structure of *Staphylococcus aureus* alpha-Toxin-Induced Ionic Channel. *Gen. Physiol. Biophys.*, 7, pp.467–473.

Kreutz, M., Ackermann, U. & Hauschildt, S., 1997. A comparative analysis of cytokine production and tolerance induction by bacterial lipopeptides, lipopolysaccharides and *Staphyloccocus aureus* in human monocytes. *Immunology*, 92, pp.396–401.

Krijgsveld, J., 2000. Thrombocidins, Microbicidal Proteins from Human Blood

Platelets, Are C-terminal Deletion Products of CXC Chemokines. *Journal of Biological Chemistry*, 275(27), pp.20374–20381.

Kroll, M.H., Harris, T.S., Moake, J.L., *et al*., 1991. von Willebrand factor binding to platelet GpIb initiates signals for platelet activation. *The Journal of clinical investigation*, 88(5), pp.1568–73.

Kubes, P., Suzuki, M. & Granger, D.N., 1991. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proceedings of the National Academy of Sciences*, 88(11), pp.4651–4655.

Kuda, O., Jenkins, C.M., Skinner, J.R., *et al*., 2011. CD36 protein is involved in store-operated calcium flux, phospholipase A2 activation, and production of prostaglandin E2. *The Journal of biological chemistry*, 286(20), pp.17785–95.

Kuharsky, A.L., & Fogelson, L., 2001. Surface-mediated control of blood coagulation: the role of binding site densities and platelet deposition. *Biophysical journal*, 80(3), pp.1050–74.

Kulkarni, S., Dopheide, S.M., Yap, C.L., *et al*., 2000. A revised model of platelet aggregation. *The Journal of clinical investigation*, 105(6), pp.783–91.

Kumar, R., Béguin, S. & Hemker, H.C., 1994. The influence of fibrinogen and fibrin on thrombin generation-evidence for feedback activation of the clotting system by clot bound thrombin. *Thrombosis and haemostasis*, 72(5), pp.713–21.

Kuwahara, M., Sugimoto, M., Tsuji, S., *et al*., 2002. Platelet shape changes and adhesion under high shear flow. *Arterioscler Thromb Vasc Biol*, 22(2),

pp.329–334.

Kwakman, P.H.S., Krijgsveld, J., De Boer, L., *et al*., 2011. Native thrombocidin-1 and unfolded thrombocidin-1 exert antimicrobial activity via distinct structural elements. *The Journal of biological chemistry*, 286(50), pp.43506–14.

Ladhani, S., 2003. Understanding the mechanism of action of the exfoliative toxins of *Staphylococcus aureus*. *FEMS Immunology & Medical Microbiology*, 39(2), pp.181–189.

Lee, B.O., Moyron-Quiroz, J., Rangel-Moreno, J., *et al*., 2003. CD40, but not CD154, expression on B cells is necessary for optimal primary B cell responses. *Journal of immunology (Baltimore, Md. : 1950)*, 171(11), pp.5707–17.

Lee, D., Fong, K.P., King, M.R., *et al*., 2012. Differential dynamics of platelet contact and spreading. *Biophysical journal*, 102(3), pp.472–82.

Lee, L.Y.L., Liang, X., Höök, M. & Brown, E.L., 2004. Identification and characterization of the C3 binding domain of the *Staphylococcus aureus* extracellular fibrinogen-binding protein (Efb). *The Journal of biological chemistry*, 279(49), pp.50710–6.

Lenz, J.C., Reusch, H.P., Albrecht, N., *et al*., 2002. Ca^{2+} controlled competitive diacylglycerol binding of protein kinase C isoenzymes in living cells. *The Journal of cell biology*, 159(2), pp.291–302.

Li, Q., Kumar, A., Gui, J.-F. & Yu, F.-S.X., 2008. *Staphylococcus aureus* lipoproteins trigger human corneal epithelial innate response through toll-like receptor-

2. *Microbial pathogenesis*, 44(5), pp.426–34.

Liu, J., Pestina, T.I., Berndt, M.C., et al., 2005. Botrocetin/VWF-induced signaling through GPIb-IX-V produces TxA2 in an alphaIIbbeta3- and aggregation-independent manner. *Blood*, 106(8), pp.2750–6.

Li, Z., Ajdic, J., Eigenthaler, M. & Du, X., 2003. A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood*, 101(11), pp.4423–4429.

Li, Z., Delaney, M.K., O'Brien, K.A. & Du, X., 2010. Signaling during platelet adhesion and activation. *Arteriosclerosis, thrombosis, and vascular biology*, 30(12), pp.2341–9.

Li, Z., Zhang, G., Feil, R., *et al*., 2006. Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alphaIIb beta3. *Blood*, 107(3), pp.965–72.

Lian, L., Wang, Y., Draznin, J., *et al*., 2005. The relative role of PLCbeta and PI3Kgamma in platelet activation. *Blood*, 106(1), pp.110–7.

Lien, E., Sellati, T.J., Yoshimura, A., *et al*., 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *The Journal of biological chemistry*, 274(47), pp.33419–33425.

Lina, G., Piémont, Y., Godail-Gamot, F., *et al*., 1999. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. *Clinical infectious diseases : an official publication*

of the Infectious Diseases Society of America, 29(5), pp.1128–32.

Lind, S.E., Yin, H.L. & Stossel, T.P., 1982. Human platelets contain gelsolin. A regulator of actin filament length. *The Journal of clinical investigation*, 69(6), pp.1384–7.

Lindemann, S., Tolley, N.D., Dixon, D. a, *et al*., 2001. Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *The Journal of cell biology*, 154(3), pp.485–90.

Litvinov, R.I., Bennett, J.S., Weisel, J.W. & Shuman, H., 2005. Multi-step fibrinogen binding to the integrin (alpha)IIb(beta)3 detected using force spectroscopy. *Biophysical journal*, 89(4), pp.2824–34.

Liu, J., Pestina, T.I., Berndt, M.C., *et al*., 2005. Botrocetin/VWF-induced signaling through GPIb-IX-V produces TxA2 in an alphaIIbbeta3- and aggregation-independent manner. *Blood*, 106(8), pp.2750–6.

Liu, J., Ware, J., Jackson, C.W. & Gartner, T.K., 2007. FcRgamma-chain signals in the absence of glycoprotein VI. *Journal of thrombosis and haemostasis : JTH*, 5(1), pp.201–3.

Locksley, R.M., 1982. Multiply Antibiotic-Resistant *Staphylococcus aureus*: Introduction, Transmission, and Evolution of Nosocomial Infection. *Annals of Internal Medicine*, 97(3), p.317.

Loppnow, H., Bil, R., Hirt, S., *et al*., 1998. Platelet-derived interleukin-1 induces cytokine production, but not proliferation of human vascular smooth muscle cells. *Blood*, 91(1), pp.134–41.

Loughman, A., Fitzgerald, J.R., Brennan, M.P., *et al*., 2005. Roles for fibrinogen, immunoglobulin and complement in platelet activation promoted by *Staphylococcus aureus* clumping factor A. *Molecular microbiology*, 57(3), pp.804–818.

Ma, D.Y. & Clark, E.A., 2010. The role of CD40 and CD40L in Dendritic Cells. *Semin Immunol*, 21(5), pp.265–272.

Ma, Y.-Q., Qin, J. & Plow, E.F., 2007. Platelet integrin alpha(IIb)beta(3): activation mechanisms. *Journal of thrombosis and haemostasis : JTH*, 5(7), pp.1345–1352.

Magwenzi, S., Woodward, C., Wraith, K.S., *et al*., 2015. Oxidized LDL activates blood platelets through CD36/NOX2-mediated inhibition of the cGMP/protein kinase G signaling cascade. *Blood*, 125(17), pp.2693–2703.

Malbec, O. & Daëron, M., 2007. The mast cell IgG receptors and their roles in tissue inflammation. *Immunological reviews*, 217, pp.206–21.

Malinin, N.L., West, X.Z. & Byzova, T. V, 2011. Oxidation as 'the stress of life'. *Aging*, 3(9), pp.906–10.

Mandell, G.L., 1975. Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. In vitro and in vivo studies with emphasis on Staphylococcal leukocyte interaction. *The Journal of clinical investigation*, 55(3), pp.561–6.

Mathias, R.A., Chen, Y.-S., Kapp, E.A., *et al*., 2011. Triton X-114 phase separation in the isolation and purification of mouse liver microsomal membrane

proteins. *Methods (San Diego, Calif.)*, 54(4), pp.396–406.

May, A., Kälsch, T., Massberg, S., *et al*., 2002. Engagement of Glycoprotein IIb/IIIa (alphaIIbbeta3) on Platelets Upregulates CD40L and Triggers CD40L-Dependent Matrix Degradation by Endothelial Cells. *Circulation*, 106(16), pp.2111–2117.

Maxeiner, H., Husemann, J., Thomas, C.A., et al., 1998. Complementary roles for scavenger receptor A and CD36 of human monocyte-derived macrophages in adhesion to surfaces coated with oxidized low-density lipoproteins and in secretion of H2O2. *J Exp Med*, 188(12), pp.2257–2265.

McAleese, F.M., Walsh, E.J., Sieprawska, M., *et al*., 2001. Loss of clumping factor B fibrinogen binding activity by *Staphylococcus aureus* involves cessation of transcription, shedding and cleavage by metalloprotease. *The Journal of biological chemistry*, 276(32), pp.29969–29978.

McCaig, L.F., McDonald, L.C., Mandal, S. & Jernigan, D.B., 2006. *Staphylococcus aureus*-associated skin and soft tissue infections in ambulatory care. *Emerging infectious diseases*, 12(11), pp.1715–23.

McCoy, K.L., Gyoneva, S., Vellano, C.P., *et al*., 2012. Protease-activated receptor 1 (PAR1) coupling to G(q/11) but not to G(i/o) or G(12/13) is mediated by discrete amino acids within the receptor second intracellular loop. *Cellular signalling*, 24(6), pp.1351–60.

McDevitt, D., Nanavaty, T., House-Pompeo, K., *et al*., 1997. Characterization of the interaction between the *Staphylococcus aureus* clumping factor (ClfA) and fibrinogen. *European journal of biochemistry / FEBS*, 247(1), pp.416–424.

McGrath, R.T., McRae, E., Smith, O.P. & O'Donnell, J.S., 2010. Platelet von Willebrand factor--structure, function and biological importance. *British journal of haematology*, 148(6), pp.834–43.

McKenzie, S.E., Taylor, S.M., Malladi, P., *et al*., 1999. The role of the human Fc receptor Fc gamma RIIA in the immune clearance of platelets: a transgenic mouse model. *Journal of immunology (Baltimore, Md. : 1950)*, 162(7), pp.4311–8.

Medina-Estrada, I., Alva-Murillo, N., López-Meza, J.E. & Ochoa-Zarzosa, A., 2015. Non-classical effects of prolactin on the innate immune response of bovine mammary epithelial cells: Implications during *Staphylococcus aureus* internalization. *Microbial pathogenesis*, 89, pp.43–53.

Mehrbod, M., Trisno, S. & Mofrad, M.R.K., 2013. On the activation of integrin αIIbβ3: outside-in and inside-out pathways. *Biophysical journal*, 105(6), pp.1304–15.

Miajlovic, H., Zapotoczna, M., Geoghegan, J. A., *et al*., 2010. Direct interaction of iron-regulated surface determinant IsdB of *Staphylococcus aureus* with the GPIIb/IIIa receptor on platelets. *Microbiology*, 156(Pt 3), pp.920–928.

Miao, W.M., Vasile, E., Lane, W.S. & Lawler, J., 2001. CD36 associates with CD9 and integrins on human blood platelets. *Blood*, 97(6), pp.1689–96.

Miethke, T., Duschek, K., Wahl, C., *et al*., 1993. Pathogenesis of the toxic shock syndrome: T cell mediated lethal shock caused by the superantigen TSST-1. *European journal of immunology*, 23(7), pp.1494–500.

Mitchell, J.A., Ali, F., Bailey, L., *et al*., 2008. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. *Experimental physiology*, 93(1), pp.141–7.

Mosesson, M., Siebenlist, K. & Meh, D., 2001. The structure and biological features of Fibrinogen and Fibrin. *Annals of the New York Academy of Sciences.*, 936, pp.11–30.

Mulcahy, M.E., Geoghegan, J.A., Monk, I.R., *et al*., 2012. Nasal Colonisation by *Staphylococcus aureus* Depends upon Clumping Factor B Binding to the Squamous Epithelial Cell Envelope Protein Loricrin A. Peschel, ed. *PLoS Pathogens*, 8(12), p.e1003092.

Müller, P., Müller-Anstett, M., Wagener, J., *et al*., 2010. The *Staphylococcus aureus* lipoprotein SitC colocalizes with toll-like receptor 2 (TLR2) in murine keratinocytes and elicits intracellular TLR2 accumulation. *Infection and Immunity*, 78(10), pp.4243–4250.

Murohara, T., Parkinson, S.J., Waldman, S.A. & Lefer, A.M., 1995. Inhibition of nitric oxide biosynthesis promotes P-selectin expression in platelets. Role of protein kinase C. *Arteriosclerosis, thrombosis, and vascular biology*, 15(11), pp.2068–75.

Moore, K.J., El Khoury, J., Medeiros, L.A., et al., 2002. A CD36-initiated signaling cascade mediates inflammatory effects of ?-amyloid. *Journal of Biological Chemistry*, 277(49), pp.47373–47379.

Naber, C.K., 2009. *Staphylococcus aureus* bacteremia: epidemiology, pathophysiology, and management strategies. *Clinical infectious diseases*,

48(4), pp.231–237.

Naik, M.U. & Naik, U.P., 2003. Calcium-and integrin-binding protein regulates focal adhesion kinase activity during platelet spreading on immobilized fibrinogen. *Blood*, 102(10), pp.3629–36.

Nakayama, H., Kurokawa, K. & Lee, B.L., 2012. Lipoproteins in bacteria: structures and biosynthetic pathways. *The FEBS journal*, 279(23), pp.4247–68.

Nergiz-Unal, R., Lamers, M.M.E., Van Kruchten, R., *et al*., 2011. Signaling role of CD36 in platelet activation and thrombus formation on immobilized thrombospondin or oxidized low-density lipoprotein. *Journal of thrombosis and haemostasis : JTH*, 9(9), pp.1835–46.

Nesbitt, W.S., Westein, E., Tovar-Lopez, F.J., *et al*., 2009. A shear gradient-dependent platelet aggregation mechanism drives thrombus formation. *Nature medicine*, 15(6), pp.665–73.

Ní Eidhin, D., Perkins, S., Francois, P., *et al*., 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. *Molecular microbiology*, 30(2), pp.245–257.

Niebuhr, M., Mühlradt, P.F., Wittmann, M., *et al*., 2008. Intracutaneous injection of the macrophage-activating lipopeptide-2 (MALP-2) which accelerates wound healing in mice a phase I trial in 12 patients. *Experimental dermatology*, 17(12), pp.1052–6.

Nieswandt, B., Aktas, B., Moers, A. & Sachs, U.J.H., 2005. Platelets in

atherothrombosis: lessons from mouse models. *Journal of thrombosis and haemostasis : JTH*, 3(8), pp.1725–36.

Nieswandt, B., Brakebusch, C., Bergmeier, W., *et al*., 2001b. Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *The EMBO journal*, 20(9), pp.2120–2130.

Nieswandt, B., Varga-Szabo, D. & Elvers, M., 2009. Integrins in platelet activation. *Journal of thrombosis and haemostasis : JTH*, 7 Suppl 1, pp.206–209.

Nieswandt, B. & Watson, S.P., 2003. Platelet-collagen interaction: is GPVI the central receptor? *Blood*, 102(2), pp.449–61.

Nonne, C., Lenain, N., Hechler, B., *et al*., 2005. Importance of platelet phospholipase C gamma 2 signaling in arterial thrombosis as a function of lesion severity. *Arteriosclerosis, thrombosis, and vascular biology*, 25(6), pp.1293–8.

Novick, R., 1967. Properties of a cryptic high-frequency transducing phage in *Staphylococcus aureus*. *Virology*, 33(1), pp.155–66.

O'Brien, L., Kerrigan, S.W., Kaw, G., *et al*., 2002. Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors ClfA and ClfB, the serine-aspartate repeat protein SdrE and protein A. *Molecular microbiology*, 44(4), pp.1033–1044.

O'Brien, L.M., Walsh, E.J., Massey, R.C., *et al*., 2002. *Staphylococcus aureus* clumping factor B (ClfB) promotes adherence to human type I cytokeratin 10: implications for nasal colonization. *Cellular microbiology*, 4(11), pp.759–

70.

O'Connell, D.P., Nanavaty, T., McDevitt, D., *et al*., 1998. The fibrinogen-binding MSCRAMM (clumping factor) of *Staphylococcus aureus* has a Ca^{2+}-dependent inhibitory site. *The Journal of biological chemistry*, 273(12), pp.6821–6829.

O'Neill, E., Pozzi, C., Houston, P., *et al*., 2008. A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB. *Journal of bacteriology*, 190(11), pp.3835–50.

O'Riordan, K. & Lee, J.C., 2004. *Staphylococcus aureus* capsular polysaccharides. *Clinical microbiology reviews*, 17(1), pp.218–34.

Oberprieler, N.G., Roberts, W., Riba, R., *et al*., 2007. cGMP-independent inhibition of integrin alphaIIbbeta3-mediated platelet adhesion and outside-in signalling by nitric oxide. *FEBS letters*, 581(7), pp.1529–34.

Offermanns, S., 2006. Activation of platelet function through G protein-coupled receptors. *Circulation research*, 99(12), pp.1293–304.

Olson, M.E. & Horswill, A.R., 2013. *Staphylococcus aureus* osteomyelitis: bad to the bone. *Cell host & microbe*, 13(6), pp.629–31.

Palabrica, T., Lobb, R., Furie, B.C., *et al*., 1992. Leukocyte accumulation promoting fibrin deposition is mediated *in vivo* by P-selectin on adherent platelets. *Nature*, 359(6398), pp.848–51.

Panigrahi, S., Ma, Y., Li, H., *et al*., 2012. Engagement of Platelet Toll-Like Receptor 9 by Novel Endogenous Ligands Promotes Platelet Hyper-Reactivity and Thrombosis. *Circulation research*, 112(1), pp.103–112.

Park, Y.M., 2014. CD36, a scavenger receptor implicated in atherosclerosis. *Experimental & molecular medicine*, 46, p.e99.

Parsonnet, J., Goering, R. V, Hansmann, M.A., *et al*., 2008. Prevalence of toxic shock syndrome toxin 1 (TSST-1)-producing strains of *Staphylococcus aureus* and antibody to TSST-1 among healthy Japanese women. *Journal of clinical microbiology*, 46(8), pp.2731–8.

Pasquet, J.M., Gross, B., Quek, L., *et al*., 1999. LAT is required for tyrosine phosphorylation of phospholipase C gamma 2 and platelet activation by the collagen receptor GPVI. *Molecular and cellular biology*, 19(12), pp.8326–34.

Patel, A.H., Nowlan, P., Weavers, E.D. & Foster, T., 1987. Virulence of protein A-deficient and alpha-toxin-deficient mutants of *Staphylococcus aureus* isolated by allele replacement. *Infection and immunity*, 55(12), pp.3103–10.

Patel, S.R., Hartwig, J.H. & Italiano, J.E., 2005. The biogenesis of platelets from megakaryocyte proplatelets. *The Journal of clinical investigation*, 115(12), pp.3348–54.

Pasquet, J.M., Gross, B., Quek, L., et al., 1999. LAT is required for tyrosine phosphorylation of phospholipase cgamma2 and platelet activation by the collagen receptor GPVI. *Molecular and cellular biology*, 19(12), pp.8326–34.

Paul, B.Z., Daniel, J.L. & Kunapuli, S.P., 1999. Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways. Role of p160 Rho-associated coiled-coil-containing protein kinase in platelet shape change. *The Journal of biological chemistry*, 274(40), pp.28293–300.

Pawar, P., Shin, P.K., Mousa, S., *et al*., 2004. Fluid shear regulates the kinetics and receptor specificity of *Staphylococcus aureus* binding to activated platelets. *Journal of immunology (Baltimore, Md. : 1950)*, 173(2), pp.1258–1265.

Payrastre, B., Missy, K., Trumel, C., *et al*., 2000. The integrin alpha IIb/beta 3 in human platelet signal transduction. *Biochemical pharmacology*, 60(8), pp.1069–74.

Pelz, A., Wieland, K.-P., Putzbach, K., *et al*., 2005. Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*. *The Journal of biological chemistry*, 280(37), pp.32493–8.

Penkett, C.J., Redfield, C., Jones, J. a, *et al*., 1998. Structural and dynamical characterization of a biologically active unfolded fibronectin-binding protein from *Staphylococcus aureus*. *Biochemistry*, 37(48), pp.17054–17067.

Pepino, M.Y., Kuda, O., Samovski, D. & Abumrad, N.A., 2014. Structure-function of CD36 and importance of fatty acid signal transduction in fat metabolism. *Annual review of nutrition*, 34, pp.281–303.

Perersen, F., Ludwig, A., Flad, H.D. & Brandt, E., 2014. TNF-alpha renders human neutrophils responsive to platelet factor 4 . Comparison of PF-4 and IL-8 reveals different activity profiles of the two PubMed Commons. *The Journal of Immunology.*, 156(5), pp.1954–1962.

Perkins, S., Walsh, E.J., Deivanayagam, C.C., *et al*., 2001. Structural organization of the fibrinogen-binding region of the clumping factor B MSCRAMM of *Staphylococcus aureus*. *The Journal of biological chemistry*, 276(48), pp.44721–44728.

Perussia, B., Trinchieri, G., Jackson, A., *et al*., 1984. The Fc receptor for IgG on human natural killer cells: phenotypic, functional, and comparative studies with monoclonal antibodies. *Journal of immunology (Baltimore, Md. : 1950)*, 133(1), pp.180–9.

Pettigrew, M.M., Gent, J.F., Revai, K., *et al*., 2008. Microbial interactions during upper respiratory tract infections. *Emerging infectious diseases*, 14(10), pp.1584–91.

Pishchany, G., Dickey, S.E. & Skaar, E.P., 2009. Subcellular localization of the *Staphylococcus aureus* heme iron transport components IsdA and IsdB. *Infection and immunity*, 77(7), pp.2624–34.

Pizzulli, L., Yang, a., Martin, J.F. & Lüderitz, B., 1998. Changes in platelet size and count in unstable angina compared to stable angina or non-cardiac chest pain. *European Heart Journal*, 19(1), pp.80–84.

Podolnikova, N.P., Yakovlev, S., Yakubenko, V.P., *et al*., 2014. The interaction of integrin αIIbβ3 with fibrin occurs through multiple binding sites in the αIIb β-propeller domain. *The Journal of biological chemistry*, 289(4), pp.2371–83.

Podrez, E.A., Febbraio, M., Sheibani, N., *et al*., 2000. Macrophage scavenger receptor CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species. *The Journal of clinical investigation*, 105(8), pp.1095–108.

Pohl, U., Nolte, C., Bunse, A., *et al*., 1994. Endothelium-dependent phosphorylation of vasodilator-stimulated protein in platelets during coronary passage. *The American journal of physiology*, 266(2 Pt 2), pp. 606–

12.

Polanowska-Grabowska, R., Gibbins, J.M. & Gear, A.R.L., 2003. Platelet adhesion to collagen and collagen-related peptide under flow: roles of the [alpha]2[beta]1 integrin, GPVI, and Src tyrosine kinases. *Arteriosclerosis, thrombosis, and vascular biology*, 23(10), pp.1934–40.

Prasad, K.S.S., Andre, P., He, M., *et al*., 2003. Soluble CD40 ligand induces beta3 integrin tyrosine phosphorylation and triggers platelet activation by outside-in signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 100(21), pp.12367–71.

Prescott, L.M., Klein, D.A. & Harley, J.P., 2002. *Microbiology*.

Pulcinelli, F.M., Pignatelli, P., Celestini, A., *et al*., 2004. Inhibition of platelet aggregation by aspirin progressively decreases in long-term treated patients. *Journal of the American College of Cardiology*, 43(6), pp.979–984.

Qi, H.Y., Sankaran, K., Gan, K. & Wu, H.C., 1995. Structure-function relationship of bacterial prolipoprotein diacylglyceryl transferase: functionally significant conserved regions. *Journal of bacteriology*, 177(23), pp.6820–4.

Ramsland, P. a, Farrugia, W., Bradford, T.M., *et al*., 2011. Structural Basis for Fc{gamma}RIIa Recognition of Human IgG and Formation of Inflammatory Signaling Complexes. *Journal of immunology (Baltimore, Md. : 1950)*, 187(6), pp.3208–3217.

Randriamboavonjy, V., Schrader, J., Busse, R. & Fleming, I., 2004. Insulin induces the release of vasodilator compounds from platelets by a nitric oxide-G

kinase-VAMP-3-dependent pathway. *The Journal of experimental medicine*, 199(3), pp.347–56.

Raslan, Z. & Naseem, K.M., 2015. Compartmentalisation of cAMP-dependent signalling in blood platelets: The role of lipid rafts and actin polymerisation. *Platelets*, 26(4), pp.349–57.

Reed, G.L., Fitzgerald, M.L. & Polgár, J., 2000. Molecular mechanisms of platelet exocytosis: insights into the 'secrete' life of thrombocytes. *Blood*, 96(10), pp.3334–3342.

Reininger, A.J., 2009. Platelet function under high shear conditions adverse shear conditions Activation-independent Platelet thrombus propagation under flow. *Hamostaseologie*, 1, pp.21–23.

Reininger, A.J., Heijnen, H.F.G., Schumann, H., *et al*., 2006. Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. *Blood*, 107(9), pp.3537–45.

Repp, R., Valerius, T., Sendler, A., *et al*., 1991. Neutrophils express the high affinity receptor for IgG (Fc gamma RI, CD64) after in vivo application of recombinant human granulocyte colony-stimulating factor. *Blood*, 78(4), pp.885–9.

Ren, Y., 2012. Peroxisome proliferator-activator receptor : A link between macrophage CD36 and inflammation in malaria infection. *PPAR Research*, 2012.

Rex, S., Beaulieu, L.M., Perlman, D.H., *et al*., 2009. Immune vs. thrombotic

stimulation of platelets differentially regulates signaling pathways, intracellular protein-protein interactions, and alpha granule release. *Thrombosis and haemostasis*, 102(1), pp.617–638.

Rigby, K.M. & DeLeo, F.R., 2012. Neutrophils in innate host defence against *Staphylococcus aureus* infections. *Seminars in immunopathology*, 34(2), pp.237–59.

Rivadeneyra, L., Carestia, A., Etulain, J., *et al*., 2014. Regulation of platelet responses triggered by Toll-like receptor 2 and 4 ligands is another non-genomic role of nuclear factor-kappaB. *Thrombosis research*, 133(2), pp.235–43.

Rivera, J., Lozano, M.L., Navarro-Núñez, L. & Vicente, V., 2009. Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica*, 94(5), pp.700–11.

Roche, F.M., Downer, R., Keane, F., *et al*., 2004. The N-terminal A domain of fibronectin-binding proteins A and B promotes adhesion of *Staphylococcus aureus* to elastin. *The Journal of biological chemistry*, 279(37), pp.38433–40.

Roest, M., Reininger, A., Zwaginga, J.J., *et al*., 2011. Flow chamber-based assays to measure thrombus formation *in vitro*: requirements for standardization. *Journal of Thrombosis and Haemostasis*, 9(11), pp.2322–2324.

Rooijakkers, S.H.M., Van Wamel, W.J.B., Ruyken, M., *et al*., 2005. Anti-opsonic properties of staphylokinase. *Microbes and infection / Institut Pasteur*, 7(3), pp.476–84.

Ruiz, F.A., Lea, C.R., Oldfield, E. & Docampo, R., 2004. Human platelet dense granules contain polyphosphate and are similar to acidocalcisomes of bacteria and unicellular eukaryotes. *The Journal of biological chemistry*, 279(43), pp.44250–7.

Sabat, A., Melles, D.C., Martirosian, G., *et al*., 2006. Distribution of the serine-aspartate repeat protein-encoding sdr genes among nasal-carriage and invasive *Staphylococcus aureus* strains. *Journal of clinical microbiology*, 44(3), pp.1135–8.

Sambrano, G.R., Weiss, E.J., Zheng, Y.W., *et al*., 2001. Role of thrombin signalling in platelets in haemostasis and thrombosis. *Nature*, 413(6851), pp.74–8.

Sánchez-Ferrer, A., Bru, R. & Garcia-Carmona, F., 1989. Novel procedure for extraction of a latent grape polyphenoloxidase using temperature-induced phase separation in Triton x-114. *Plant physiology*, 91(4), pp.1481–7.

Sankaran, K. & Wu, H.C., 1994. Lipid modification of bacterial prolipoprotein. Transfer of diacylglyceryl moiety from phosphatidylglycerol. *The Journal of biological chemistry*, 269(31), pp.19701–19706.

Sannasiddappa, T.H., Costabile, A., Gibson, G.R. & Clarke, S.R., 2011. The influence of *Staphylococcus aureus* on gut microbial ecology in an *in vitro* continuous culture human colonic model system. *PloS one*, 6(8), p.e23227.

Savage, B., Saldívar, E. & Ruggeri, Z.M., 1996. Initiation of platelet adhesion by arrest onto fibrinogen or translocation on von Willebrand factor. *Cell*, 84(2), pp.289–97.

Savage, B., Sixma, J.J. & Ruggeri, Z.M., 2002. Functional self-association of von Willebrand factor during platelet adhesion under flow. *Proceedings of the National Academy of Sciences of the United States of America*, 99(1), pp.425–30.

Schmaler, M., Jann, N.J., Ferracin, F., *et al*., 2009. Lipoproteins in *Staphylococcus aureus* mediate inflammation by TLR2 and iron-dependent growth in vivo. *Journal of immunology (Baltimore, Md. : 1950)*, 182(11), pp.7110–8.

Schmitt, A., Guichard, J., Massé, J.-M., *et al*., 2001. Of mice and men: Comparison of the ultrastructure of megakaryocytes and platelets. *Experimental Hematology*, 29(11), pp.1295–1302.

Schneider, S.W., Nuschele, S., Wixforth, A., *et al*., 2007. Shear-induced unfolding triggers adhesion of von Willebrand factor fibers. *Proceedings of the National Academy of Sciences of the United States of America*, 104(19), pp.7899–903.

Schober, A., Manka, D., Hundelshausen, P. von, *et al*., 2002. Deposition of Platelet RANTES Triggering Monocyte Recruitment Requires P-Selectin and Is Involved in Neointima Formation After Arterial Injury. *Circulation*, 106(12), pp.1523–1529.

Schröder, A., Schröder, B., Roppenser, B., *et al*., 2006. *Staphylococcus aureus* fibronectin binding protein-A induces motile attachment sites and complex actin remodeling in living endothelial cells. *Molecular biology of the cell*, 17(12), pp.5198–210.

Schubert, S., Schwertz, H., Weyrich, A.S., *et al*., 2011. *Staphylococcus aureus* α-

Toxin Triggers the Synthesis of B-Cell Lymphoma 3 by Human Platelets. *Toxins*, 3(2), pp.120–133.

Sedlmayr, P., Blaschitz, A., Wilders-Truschnig, M., *et al*., 1995. Platelets contain interleukin-1 alpha and beta which are detectable on the cell surface after activation. *Scandinavian journal of immunology*, 42(2), pp.209–14.

Senis, Y. a, Mazharian, A. & Mori, J., 2015. Review Article Src family kinases : at the forefront of platelet activation. *Blood*, 124(13), pp.2013–2025.

Shannon, O. & Flock, J.-I., 2004. Extracellular fibrinogen binding protein, Efb, from *Staphylococcus aureus* binds to platelets and inhibits platelet aggregation. *Thrombosis and haemostasis*, 91(4), pp.779–89.

Sheldon, J.R. & Heinrichs, D.E., 2012. The iron-regulated Staphylococcal lipoproteins. *Frontiers in cellular and infection microbiology*, 2(April), p.41.

Shido, K., Ahmad, G., Hsu, L. & Kamiyama, M., 1995. Characterization of human platelet IgG Fc receptor associated with membrane glycoprotein. *Journal of clinical & laboratory immunology*, 46(1), pp.1–11.

Shiraki, R., Inoue, N., Kawasaki, S., *et al*., 2004. Expression of Toll-like receptors on human platelets. *Thromb Res*, 113(6), pp.379–385.

Siboo, I.R., Chambers, H.F. & Sullam, P.M., 2005. Role of SraP, a Serine-Rich Surface Protein of *Staphylococcus aureus*, in binding to human platelets. *Infection and immunity*, 73(4), pp.2273–2280.

Signas, C., Rauccit, G., Jonsson, K., *et al*., 1989. Nucleotide sequence of the gene for a fibronectin-binding protein from *Staphylococcus aureus* : Use of this

peptide sequence in the synthesis of biologically active peptides. *Proceedings of the National Academy of Sciences of the United States of America*, 86(January), pp.699–703.

Silverstein, R.L. & Febbraio, M., 2009. CD36, a scavenger receptor involved in immunity, metabolism, angiogenesis, and behavior. *Science signaling*, 2(72).

Silverstein, R.L., Li, W., Park, Y.M. & Rahaman, S.O., 2010. Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. *Transactions of the American Clinical and Climatological Association*, 121, pp.206–20.

Sinha, a K., Rao, a K., Willis, J. & Colman, R.W., 1983. Inhibition of thromboxane A2 synthesis in human platelets by coagulation factor Xa. *Proceedings of the National Academy of Sciences of the United States of America*, 80(19), pp.6086–90.

Smethurst, P. a, Onley, D.J., Jarvis, G.E., *et al*., 2007. Structural basis for the platelet-collagen interaction: the smallest motif within collagen that recognizes and activates platelet Glycoprotein VI contains two glycine-proline-hydroxyproline triplets. *The Journal of biological chemistry*, 282(2), pp.1296–304.

Smyth, S.S., McEver, R.P., Weyrich, A.S., *et al*., 2009. Platelet functions beyond hemostasis.. *Journal of thrombosis and haemostasis : JTH*, 7(11), pp.1759–66.

Soto, A.G., Smith, T.H., Chen, B., *et al*., 2015. N-linked glycosylation of protease-activated receptor-1 at extracellular loop 2 regulates G-protein signaling bias. *Proceedings of the National Academy of Sciences*, p.201508838.

Souli, M. & Giamarellou, H., 1998. Effects of Slime produced by clinical isolates of Coagulase-negative Staphylococci on activities of various antimicrobial agents. *Antimicrob. Agents Chemother.*, 42(4), pp.939–941.

Stark, R.J., Aghakasiri, N. & Rumbaut, R.E., 2012. Platelet-Derived Toll-Like Receptor 4 (Tlr-4) Is Sufficient to Promote Microvascular Thrombosis in Endotoxemia. *PLoS ONE*, 7(7), p.e41254.

Stenberg, P.E., Shuman, M.A., Levine, S.P. & Bainton, D.F., 1984. Redistribution of alpha granules and their contents in thrombin-stimulated platelets. *Journal of Cell Biology*, 98(February), pp.748–760.

Stoll, H., Dengjel, J., Nerz, C. & Götz, F., 2005. *Staphylococcus aureus* deficient in lipidation of prelipoproteins is attenuated in growth and immune activation. *Infection and immunity*, 73(4), pp.2411–23.

Stewart, C.R., Stuart, L.M., Wilkinson, K., et al., 2010. UKPMC Funders Group a Toll-like receptor 4 and 6 heterodimer. *Nature Immunology*, 11(2), pp.155–161.

Stuart, L.M., Deng, J., Silver, J.M., et al., 2005. Response to Staphylococcus aureus requires CD36-mediated phagocytosis triggered by the COOH-terminal cytoplasmic domain. *The Journal of cell biology*, 170(3), pp.477–85.

Sullam, P.M., Bayer, a S., Foss, W.M. & Cheung, a L., 1996. Diminished platelet binding in vitro by *Staphylococcus aureus* is associated with reduced virulence in a rabbit model of infective endocarditis. *Infection and immunity*, 64(12), pp.4915–4921.

Sundgren, N.C., Zhu, W., Yuhanna, I.S., *et al*., 2011. Coupling of Fcγ receptor I to Fcγ receptor IIb by SRC kinase mediates C-reactive protein impairment of endothelial function. *Circulation research*, 109(10), pp.1132–40.

Surewaard, B.G.J., De Haas, C.J.C., Vervoort, F., *et al*., 2013. Staphylococcal alpha-phenol soluble modulins contribute to neutrophil lysis after phagocytosis. *Cellular microbiology*, 15(8), pp.1427–37.

Sutcliffe, I.C. & Russell, R., 1995. Lipoproteins of Gram-Positive Bacteria. *Journal of Bacteriology*, 177(5), pp.1123–1128.

Tager, M., 1974. Current views on the mechanisms of coagulase action in blood clotting? *Annals of the New York Academy of Sciences*, 236(1 Recent Advanc), pp.277–291.

Taub, R., Gould, R.J., Garsky, V.M., *et al*., 1989. A monoclonal antibody against the platelet fibrinogen receptor contains a sequence that mimics a receptor recognition domain in fibrinogen. *The Journal of biological chemistry*, 264(1), pp.259–65.

Tawaratsumida, K., Furuyashiki, M., Katsumoto, M., *et al*., 2009. Characterization of N-terminal structure of TLR2-activating lipoprotein in *Staphylococcus aureus*. *The Journal of biological chemistry*, 284(14), pp.9147–9152.

Thammavongsa, V., Missiakas, D.M. & Schneewind, O., 2013. *Staphylococcus aureus* degrades neutrophil extracellular traps to promote immune cell death. *Science (New York, N.Y.)*, 342(6160), pp.863–6.

Thorne, R.F., Marshall, J.F., Shafren, D.R., *et al*., 2000. The integrins alpha3beta1

and alpha6beta1 physically and functionally associate with CD36 in human melanoma cells. Requirement for the extracellular domain of CD36. *The Journal of biological chemistry*, 275(45), pp.35264–75.

Tousoulis, D., Kampoli, A.-M., Tentolouris, C., *et al*., 2012. The role of nitric oxide on endothelial function. *Current vascular pharmacology*, 10(1), pp.4–18.

Tsuboi, S., 2002. Calcium integrin-binding protein activates platelet integrin alpha IIbbeta 3. *The Journal of biological chemistry*, 277(3), pp.1919–23.

Triantafilou, M., Gamper, F.G.J., Haston, R.M., et al., 2006. Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and intracellular targeting. *The Journal of biological chemistry*, 281(41), pp.31002–11.

Valaperta, R., Tejada, M.R., Frigerio, M., *et al*., 2010. *Staphylococcus aureus* nosocomial infections: the role of a rapid and low-cost characterization for the establishment of a surveillance system. *The new microbiologica*, 33(3), pp.223–32.

Valiyaveettil, M., Kar, N., Ashraf, M.Z., Byzova, T. V, Febbraio, M. & Podrez, E. a, 2008. Oxidized high-density lipoprotein inhibits platelet activation and aggregation via scavenger receptor BI. *Blood*, 111(4), pp.1962–71.

Valiyaveettil, M., Kar, N., Ashraf, M.Z., Byzova, T. V, Febbraio, M. & Podrez, E.A., 2008. Oxidized high-density lipoprotein inhibits platelet activation and aggregation via scavenger receptor BI. *Blood*, 111(4), pp.1962–71.

Valiyaveettil, M. & Podrez, E.A., 2009. Platelet hyperreactivity, scavenger

receptors and atherothrombosis. *Journal of thrombosis and haemostasis : JTH*, 7 Suppl 1, pp.218–21.

Vanassche, T., Kauskot, A., Verhaegen, J., *et al*., 2011. Fibrin formation by staphylothrombin facilitates *Staphylococcus aureus* -induced platelet aggregation. *Thrombosis and haemostasis*, 107(6), pp.1107–1121.

Varga-Szabo, D., Braun, A. & Nieswandt, B., 2009. Calcium signaling in platelets. *Journal of thrombosis and haemostasis : JTH*, 7(7), pp.1057–66.

Varga-Szabo, D., Pleines, I. & Nieswandt, B., 2008. Cell adhesion mechanisms in platelets. *Arteriosclerosis, thrombosis, and vascular biology*, 28(3), pp.403–412.

Vassbotn, F.S., Havnen, O.K., Heldin, C.H. & Holmsen, H., 1994. Negative feedback regulation of human platelets via autocrine activation of the platelet-derived growth factor alpha-receptor. *The Journal of biological chemistry*, 269(19), pp.13874–9.

Vitrat, N., Cohen-Solal, K., Pique, C., *et al*., 1998. Endomitosis of human megakaryocytes are due to abortive mitosis. *Blood*, 91(10), pp.3711–23.

Wagner, S.J., 1996. CD36 Is Palmitoylated on Both N- and C-terminal Cytoplasmic Tails. *Journal of Biological Chemistry*, 271(37), pp.22315–22320.

Wahlsten, J.L. & Ramakrishnan, S., 1998. Separation of function between the domains of toxic shock syndrome toxin-1. *Journal of immunology (Baltimore, Md. : 1950)*, 160(2), pp.854–9.

Waller, A.K., Sage, T., Kumar, C., *et al*., 2013. *Staphylococcus aureus* lipoteichoic

acid inhibits platelet activation and thrombus formation via the Paf receptor. *The Journal of infectious diseases*, 208(12), pp.2046–57.

Wang, G.R., Zhu, Y., Halushka, P. V, *et al*., 1998. Mechanism of platelet inhibition by nitric oxide: in vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase. *Proceedings of the National Academy of Sciences of the United States of America*, 95(9), pp.4888–4893.

Ware, J., 2004. Dysfunctional platelet membrane receptors: from humans to mice. *Thrombosis and haemostasis*, 92(3), pp.478–85.

Watson, S.P. & Gibbins, J., 1998. Collagen receptor signalling in platelets: extending the role of the ITAM. *Immunology today*, 19(6), pp.260–4.

Watson, S.P., Herbert, J.M.J. & Pollitt, a Y., 2010. GPVI and CLEC-2 in hemostasis and vascular integrity. *Journal of thrombosis and haemostasis : JTH*, 8(7), pp.1456–67.

Watson, S.P., Auger, J.M., McCarty, O.J.T. & Pearce, a C., 2005. GPVI and integrin alphaIIb beta3 signaling in platelets. *Journal of thrombosis and haemostasis : JTH*, 3(8), pp.1752–62.

Weidenmaier, C., Peschel, A., Xiong, Y.-Q., *et al*., 2005. Lack of wall teichoic acids in *Staphylococcus aureus* leads to reduced interactions with endothelial cells and to attenuated virulence in a rabbit model of endocarditis. *The Journal of infectious diseases*, 191(10), pp.1771–7.

Weinstein, L., 1938. The Staphylococci of the Human Vagina. *The Yale journal of biology and medicine*, 10(5), pp.519–25.

Weisel, J.W., 2007. Structure of fibrin: impact on clot stability. *Journal of thrombosis and haemostasis : JTH*, 5 Suppl 1, pp.116–24.

Weyrich, A.S. & Zimmerman, G. a, 2004. Platelets: signaling cells in the immune continuum. *Trends in immunology*, 25(9), pp.489–495.

Whiteheart, S.W., 2011. Platelet granules: surprise packages. *Blood*, 118(5), pp.1190–1.

Wiseman, G.M., 1975. The hemolysins of *Staphylococcus aureus*. *Bacteriological reviews*, 39(4), pp.317–344.

Wolberg, A.S. & Campbell, R.A., 2008. Thrombin generation, fibrin clot formation and hemostasis. *Transfusion and Apheresis Science*, 38(1), pp.15–23.

Worth, R.G., Chien, C.D., Chien, P., *et al*., 2006. Platelet FcgammaRIIA binds and internalizes IgG-containing complexes. *Experimental hematology*, 34(11), pp.1490–5.

Woulfe, D., Jiang, H., Mortensen, R., *et al*., 2002. Activation of Rap1B by Gi Family Members in Platelets. *Journal of Biological Chemistry*, 277(26), pp.23382–23390.

Wu, B.-Q., Zhi, M.-J., Liu, H., *et al*., 2011. Inhibitory effects of lipoteichoic acid from *Staphylococcus aureus* on platelet function and platelet-monocyte aggregation. *Inflammation research : official journal of the European Histamine Research Society*, 60(8), pp.775–82.

Wu, J., 1995. Recombinant GST/CD36 Fusion Proteins Define a Thrombospondin Binding Domain. *Journal of Biological Chemistry*, 270(7), pp.2981–2986.

Xiang, H., Feng, Y., Wang, J., *et al*., 2012. Crystal structures reveal the multi-ligand binding mechanism of *Staphylococcus aureus* ClfB. *PLoS pathogens*, 8(6), p.e1002751.

Yacoub, D., Theoret, J.F., Villeneuve, L., *et al*., 2006. Essential Role of Protein Kinase C δ in Platelet Signaling, αIIbβ3 Activation, and Thromboxane A2 Release. *J Biol Chem*, 281(40), pp.30024–30035.

Yoshida, K., Asaoka, Y. & Nishizuka, Y., 1992. Platelet activation by simultaneous actions of diacylglycerol and unsaturated fatty acids. *Proceedings of the National Academy of Sciences of the United States of America*, 89(14), pp.6443–6.

Yves, L.L., Florence, B. & Michel, G., 2003. *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res.*, 2(1), pp.63–76.

Zawrotniak, M. & Rapala-Kozik, M., 2013. Neutrophil extracellular traps (NETs) - Formation and implications. *Acta Biochimica Polonica*, 60(3), pp.277–284.

Zellweger, R.M. & Shresta, S., 2014. Mouse models to study dengue virus immunology and pathogenesis. *Frontiers in immunology*, 5, p.151.

Zhi, H., Dai, J., Liu, J., *et al*., 2015a. Platelet Activation and Thrombus Formation over IgG Immune Complexes Requires Integrin αIIbβ3 and Lyn Kinase K. Freson, ed. *PLOS ONE*, 10(8), p.e0135738.

Zhi, H., Rauova, L., Hayes, V., *et al*., 2013. Cooperative integrin/ITAM signaling in platelets enhances thrombus formation in vitro and in vivo. *Blood*, 121(10), pp.1858–67.

Zingg, J.M., Ricciarelli, R. & Azzi, A., 2000. Scavenger receptors and modified lipoproteins: fatal attractions? *IUBMB life*, 49(5), pp.397–403.

Zucker-Franklin, D. & Philipp, C.S., 2000. Platelet production in the pulmonary capillary bed: new ultrastructural evidence for an old concept. *The American journal of pathology*, 157(1), pp.69–74.

Zurbano, M.J., Fusté, B., Arderiu, G., *et al*., 2003. Differences and similarities in tyrosine phosphorylation of proteins in platelets from human and pig species. *Journal of thrombosis and haemostasis : JTH*, 1(11), pp.2411–8.