The effects of *Staphylococcus aureus* Phenol-Soluble Modulin α3 on Human Platelets

By

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Declaration

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

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Abstract

Staphylococcus aureus is a major opportunistic pathogenic bacterium which is known to interact with human platelets and modulate their function. Community-Acquired Methicillin-resistant *Staphylococcus aureus* (CA-MRSA) strains are serious human pathogens because of their ability to resist and evade the host immune defences and are commonly resistant to clinically important antibiotics. In the last decade, CA-MRSA infection has become a major worldwide problem and causes a significant increase in the rate of human morbidity and mortality. CA-MRSA can cause acute infections such as sepsis, meningitis and endocarditis due to the production of a class of toxins called phenol-soluble modulins (PSMs) which are considered as important virulence factors. PSM toxins play important roles in the pathogenesis of *S. aureus* through interaction with immune cells of the host and modulation of their functions.

The aims of this study were to determine the effect of *S. aureus* phenol-soluble modulin α3 (PSMα3) toxin on human platelet activity, what effect *S. aureus* PSMα3 has on platelet functions and to identify the mechanism of interaction between *S. aureus* PSMα3 and human platelets. Functional studies showed *S. aureus* PSMα3 toxin to be an inhibitor of platelet activation. This inhibitory effect of *S. aureus* PSMα3 was observed with inhibition of platelet aggregation mediated by physiological agonists and *S. aureus* cells. The toxin caused a reduction in the activation of integrin $\alpha_{IIb}\beta_3$ and inhibited spreading and thrombus formation. VPAC1 was identified as the major platelet surface receptor interacting with *S. aureus* PSMα3. Pre-incubation of platelets with PSMα3 resulted in increased levels of cAMP and consequent activation of protein kinase A (PKA).

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List of Abbreviations

ABC	ATP-binding cassette transporters
ACD	Acid citrate dextrose
ACs	Adenylyl cyclases
ADP	Adenosine diphosphate
agr	Accessory gene regulator
AMPs	Antimicrobial peptides
ANOVA	Analysis of Variance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CA-MRSA	Community-acquired methicillin-resistant *S. aureus*
cGMP	Cyclic guanosine monophosphate
CHIPS	Chemotaxis inhibitory protein of *S. aureus*
CLEC-2	C-type lectin like receptor 2
ClfA	Clumping factor A
CRP-XL	Collagen-related-peptide-cross-linked
CT	Cholera toxin
DAG	Diacylglycerol
DCs	Dendritic cells
ddH_2O	Double-distilled water
DIOC6	3,3-dihexyloxacarbocyanine iodide
DTS	Dense tubular system

ECL	Enhanced chemiluminescence
Efb	Extracellular fibrinogen binding proteins
ELISA	Enzyme-linked immunosorbent assay
FcRγ	Fc receptor gamma chain
FLIPr	FPR2 inhibitory protein
FnBPA	Fibronectin binding protein A
FnBPB	Fibronectin binding protein B
FPR1	Formyl peptide receptor 1
FPR2	Formyl peptide receptor 2
FPR3	Formyl peptide receptor 3
GPCRs	G protein-coupled receptors
GPIb	Glycoprotein Ib
GPRP	Gly-Pro-Arg-Pro
GPVI	Glycoprotein VI
HA-MRSA	Healthcare-acquired MRSA
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP3	Inositol trisphosphate
IsdA	Iron-regulated surface determinant A
LAMP-1	Lysosomal-associated membrane protein 1
LAMP-2	Lysosomal-associated membrane protein 2
LAMP-3	Lysosomal-associated membrane protein 3
LTA	Lipoteichoic acid
MGEs	Mobile genetic element
MRSA	Methicillin-resistant *Staphylococcus aureus*

MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NO	Nitric oxide
ºC	Degree Celsius
OCS	Open canalicular system
OD	Optical density
PafR	Platelet activating factor receptor
PAR1	Protease-activated receptor 1
PAR4	Protease-activated receptor 4
PBS	Phosphate-buffered saline
PF4	Platelet factor 4
PGI_2	Prostaglandin I_2
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
Pmt	Phenol-soluble modulin transporter
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PSGL-1	P-selectin glycoprotein ligand-1
PSMα3	Phenol-soluble modulin α 3
PT	Pertussis toxin
PVDF	Polyvinylidene difluoride
RANTES	regulated on activation normal T expressed and secreted
S. aureus	*Staphylococcus aureus*

IE	Infective endocarditis
SCCmec	Staphylococcal cassette chromosome mec
SdrC	Serine-aspartate repeat-containing protein C
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sGC	Soluble guanyle cyclase
TBS-T	Tris-buffered saline (TBS) and Tween 20
TEMED	Tetramethylethylenediamine
TLR2	Toll-like receptor 2
TXA_2	Thromboxane A_2
VPAC1	Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor 1
vWF	Von Willebrand factor
WRW4	WRWWWW
WTA	Wall teichoic acid

CHAPTER 1

Introduction

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1. Introduction

1.1 Staphylococci

Staphylococci were discovered by Sir Alexander Ogston in 1880 (Archer, 1998) and described as Gram-positive, spherical and non-motile bacteria (Harris *et al.*, 2002; Hennekinne *et al.*, 2012). The nutritional requirements for these microorganisms are different between species, but there are 5-12 amino acids such as nicotinamide, vitamin B and thiamine that are common nutritional sources (Harris *et al.*, 2002).

Important features of these bacteria include their ability to resist heat and tolerate high salt. Staphylococci are catalase-positive and oxidase-negative which differentiates them from other Gram-positive bacteria, such as streptococci (Lowy, 1998). *Staphylococcus aureus* is an important member of the phylum due to its burden of human diseases (Hennekinne *et al.*, 2012).

S. aureus is a ubiquitous bacterium, found in various environments such as soil, contaminated food, human and animal skin and the anterior nares of human beings (Lowy, 1998). The microscopic analysis of this bacterium shows it to be arranged in grape-like clusters, with cells having a diameter of 0.5–1.5 μm (Kluytmans *et al.*, 1997). This bacterium can be distinguished from many other staphylococcal species by its coagulase production and its yellowish appearance on mannitol salt agar (Lowy, 1998).

1.2 *S. aureus* infections

S. aureus is contagious and can be spread from person to person by touching, sneezing and coughing (Graham *et al.*, 2006). This bacterium is estimated to be carried by 2 billion people worldwide and 53 million of those people are infected with a methicillin-resistant *Staphylococcus aureus* (MRSA) (Graham *et al.*, 2006) which is commonly resistant to multiple antibiotics, making its infections difficult to treat (Graham *et al.*, 2006). In 2007, the Centre for Disease Control in Atlanta (CDC) reported that the numbers of deaths in USA due to MRSA were higher than those caused by human immunodeficiency viruses (HIV) (Courvalin *et al*., 2011).

MRSA was once confined to healthcare settings through colonisation with healthcare-acquired methicillin-resistant *S. aureus* (HA-MRSA) strains (David *et al.*, 2010). However, evolution of MRSA occurred in the mid-1990s resulting in increased numbers of MRSA infections among the community (Li *et al.,* 2009). This explosion in the number of MRSA infections was due to the appearance of new strains of MRSA, which were named as community-acquired methicillin-resistant *S. aureus* (CA-MRSA). In 1999, CA-MRSA infections were reported in different cities in the USA and Western Australia (Mediavilla *et al.*, 2012). In 2000, CA-MRSA was characterised as a global epidemic due to several reports from many countries (Mediavilla *et al.*, 2012) and the CDC defined CA-MRSA infections as MRSA infections that occur among individuals in communities who are non-exposed to health care environments (David *et al.*, 2010). CA-MRSA and HA-MRSA are different; CA-MRSA resists to ciprofloxacin while HA-MRSA is sensitive to it. HA-MRSA strains frequently lack α-hemolysin, Panton-Valentine leukocidin (PVL) and exfoliative toxin (Gillen *et al.*, 2015), while CA-MRSA contains these toxins (Gillen *et al.*, 2015). CA-MRSA and HA-MRSA also have clinical

differences. For example, CA-MRSA enters the bloodstream through surgical sites, lungs and implant sites, while HA-MRSA affects postsurgical patients, dialysis patients and people with recent hospitalisations (Gillen *et al.*, 2015). CA-MRSA are often hyper-virulent strains of *S. aureus* that cause significant morbidity and mortality due to their multi-drug resistant nature, unique pattern of virulence factor production and an ability to cause serious infections in humans (Wang *et al.*, 2007).

1.3 Pathogenesis of *S. aureus*

S. aureus is one of the most common human pathogenic bacteria and in involved in food poisoning, wound infection, sepsis and infective endocarditis (IE) (Razavi *et al.*, 2014). This bacterium secretes toxins that cause disruption of host tissue and cause both superficial and deep infections.

IE occurs when the heart valves become colonised by the pathogen (Sullam *et al.*, 1996). The disease occurs when *S. aureus* enters the bloodstream and replicates. *S. aureus* attaches to circulating platelets and gains access to the heart valves. Upon attachment of *S. aureus* to the surface of heart valves and causes vegetation formation on the endothelium of the heart valves (Figure 1.1).

Currently, IE is treatable, but is still correlated with a high rate of morbidity and mortality (Habib *et al*., 2009). The mortality rate of IE in hospital admitted patients who survive the active phase of IE, which involves persistent signs of septicaemia in spite of antibiotic therapy and congestive heart failure, is between 14-22% (Fowler *et al*., 2005; Murdoch *et al*., 2009; Thuny *et al*., 2012). The 1-year mortality rate increases to 40% (Fowler *et al*., 2005; Murdoch *et al*., 2009; Thuny *et al*., 2012) and the 2-year mortality rate

approaches an average of 66% (Sohail *et al*., 2007). On the other hand, the 2-year mortality reduces to 18% if the infected device is removed and the patient receives antimicrobial therapy (Sohail *et al*., 2007).

Clinical presentation of IE may include symptoms such as fever, heart murmur and hematuria (Habib *et al.*, 2009), but fever is the most common symptom in these patients (Mylonakis *et al.*, 2001). Temperature rarely exceeds 39.4^{o}C in young patients, while elderly patients may have no fever (Mylonakis & Calderwood, 2001). Heart murmers are common associated with around 85% of IE patients (Sexton *et al.*, 2003). Heart murmers, are an abnormal heart sound, caused by turbulence of blood flowing in the heart chambers (Sexton & Spelman, 2003). The disease can lead to dysfunction of heart valves and complete hemodynamic failure (Habib *et al.,* 2009), explaining why the disease has such a high mortality rate (Habib *et al.,* 2009). Hematuria presents in patients with IE due to kidney damage (Demin *et al.*, 1991). Patients with IE may present with clinical signs such as Osler's nodes, splinter hemorrhages and splenomegaly (Habib *et al.,* 2009). Osler's nodes are painful lesions present on the hands and feet (Yee *et al.*, 1987), which are formed due to immune complexes deposition that leads to triggering of inflammatory responses causing redness, swelling and painful lesions (Farrior *et al.*, 1976). Moreover, tiny blood clots appear under the nails, known as splinter hemorrhages, in around 15% of patients with IE (Habib *et al.,* 2009).

Management of IE requires high doses of antibiotics to be given intravenously for 2 to 6 weeks (Habib *et al.,* 2009). In acute IE, treatment starts immediately after blood culture, with antibiotics including vancomycin, ceftriaxone and aminoglycoside being given while waiting for microbial diagnosis (Habib *et al.,* 2009). Upon identification of the

offending microorganism, antimicrobial therapy changes to target the identified pathogen (Habib *et al.,* 2009).

There are two important steps in development of IE. First, bacteria attach to a nidus present in the heart to start the pathogenic process. This nidus can be intra-cardiac prosthetic material, inflammation or damaged endothelium (Pappelbaum *et al.*, 2013). Second, bacteria gain access to the blood and flows through the heart, then bacteria to attach to nidus (Pappelbaum *et al.,* 2013).

Platelet activation and endothelial cells are key components in IE. Activation and aggregation of platelets is promoted by multiple virulence factors of *S. aureus* such as SpA, EAP and FnBP. SpA binds von Willebrand Factor (vWF) that has an important role in pathogenesis of IE (Hartleib *et al.,* 2000). Adhesion of vWF to platelet plays a role in adhesion of *S. aureus* to damaged endothelium that leads to growth of the infective vegetation (Hartleib *et al.*, 2000). vWF is a large glycoprotein that binds to GPIb-V-IX complex on the surface of platelets. SpA can indirectly activate platelets via binding to regions in helices I and II in vWF (Hartleib *et al.,* 2000). Thus, leading to activation of platelets and growth of the infective vegetation (Hartleib *et al.,* 2000).

Besides SpA, *S aureus* also secretes extracellular adherence protein (Eap) that causes activation of platelets (Bertling *et al.*, 2012). This platelet activation occurs through direct binding of Eap to glycosaminoglycans on the surface of platelets that leads to stimulate thiol isomerases (Bertling *et al.,* 2012; Heptinstall, 2012). Eap also bind to peripheral

blood mononuclear cells that triggers release of TNF-α. Increased TNFα levels enhance adhesion of bacteria to endothelial cells via SpA (Edwards *et al.*, 2012).

Furthermore, *S aureus* also produces fibronectin binding proteins (FnBPA and FnBPB) that can assist interaction of bacteria with the cardiac endothelium (Schröder *et al.*, 2006). FnBPA forms a fibronectin bridge between *S. aureus* and integrin $\alpha_5\beta_1$ on endothelial cells that allows the bacteria to invade cardiac endothelial cells (Schröder *et al.,* 2006). FnBPA and FnBPB also can bind to platelets through the formation of a bridge between fibrinogen or fibronectin with integrin $\alpha_{IIb}\beta_3$ on the surface of platelets. Consequently, platelets become aggregated leading to formation of an infective thrombus on the surface of heart valves (Fitzgerald *et al.*, 2006).

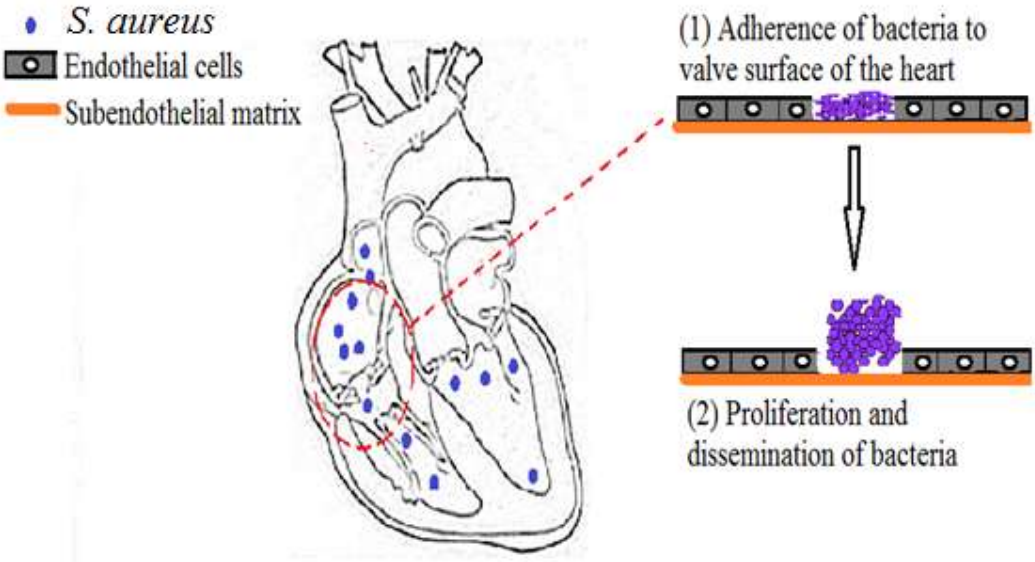

Figure 1.1: The mechanism of IE. *S. aureus* gains access to the bloodstream. Bacteria attach to circulating platelets and gain access to the heart valves, hence the pathogen attaches to the surface of heart valves (1). *S. aureus* interacts with platelets that leading them to become activated. Thus, a vegetation is formed on the endothelium of the heart valves (2). Figure adapted from Werdan *et al*., (2014).

S. aureus infection is a three stage process. First, the pathogen must colonise the host, and then the bacterium must invade and evade the defences before it is able to cause disease. Colonisation of *S. aureus* starts through interaction of microbial surface components recognising adhesive matrix molecules (MSCRAMMs) with host cells and extracellular milieu or serum proteins (Krishna *et al.*, 2012).

1.3.1 MSCRAMMs

MSCRAMMs are surface proteins expressed by *S. aureus* that are able to interact with serum and extracellular matrix molecules (McDevitt *et al.*, 1995; Wann *et al.*, 2000). Some of these surface proteins are linked to the cell wall of *S. aureus* by a sortase mediated LPXTG motif (Leu-Pro-any-Thr-Gly) (Bentley *et al.*, 2008). The C-terminal motif of these proteins is recognised by sortase A (Mazmanian *et al.*, 1999).

Clumping factors A and B (ClfA and ClfB) and fibronectin binding proteins (FnBPA and FnBPB) are MSCRAMM molecules which are anchored to the cell wall of *S. aureus* (McDevitt *et al.*, 1995; O'Brien *et al.*, 2002; Weidenmaier *et al.*, 2004). ClfA and ClfB play a role in binding to fibrinogen through their R domain that displays the fibrinogen binding A domain (Hartford *et al.*, 1997). ClfA has a vital role in causing endocarditis. ClfA mutants show decreased pathogenicity of the bacterium in an endocarditis experimental model in rabbits and rats (Moreillon *et al.*, 1995; Sullam *et al.*, 1996).

FnBPA binds to fibronectin on multiple cells of the host and forms a bridge of fibronectin between *S. aureus* and integrin $\alpha_5\beta_1$ on endothelial cells. This interaction allows the bacteria to invade nonprofessional phagocytic cells (Schröder *et al.*, 2006).

Serine-aspartate repeat-containing protein C (SdrC) is an important type of Sdr protein that is secreted by *S. aureus* (Sabat *et al.*, 2006). SdrC can adhere to β-neurexin that is an important molecule in synapse function (Barbu *et al.*, 2010). This interaction of SdrC with β-neurexin may contribute to polyneuropathy associated with sepsis (Barbu *et al.*, 2010). Iron-regulated surface determinant A (IsdA) has a role in binding to fibrinogen, fibronectin and cytokeratin K10 of epithelial cells (Clarke *et al.*, 2009). Wall teichoic acid (WTA) is an important cell wall molecule that assists colonisation of *S. aureus* to nasal epithelial cells *via* specific interaction with host lectin-like receptors (Weidenmaier *et al.*, 2004).

1.3.2 Extracellular molecules

Several molecules of *S. aureus* such as secreted toxins and ligand-binding proteins that release into the extracellular milieu also play different roles in the pathogenesis of this bacterium (Moreillon *et al.*, 2004).

1.3.2.1 Extracellular fibrinogen binding protein

During the post-exponential phase of *S. aureus* growth, extracellular fibrinogen binding protein (Efb) is produced in high concentrations (Bodén Wästfelt *et al.*, 1995). Efb has a role in immune evasion of *S. aureus* (Ko *et al.*, 2011). Efb has anti-thrombotic properties that inhibit platelet aggregation. The binding site for fibrinogen is located on the N-terminal region of Efb (Shannon *et al.*, 2004).

1.3.2.2 α-toxin, Exfoliative toxins and Toxic shock syndrome toxin

S. aureus produces α-toxin that has a strong effect on the host *via* binding to ADAM10 receptor of host cells (Menestrina *et al.*, 2001; Wilke *et al.*, 2010). This toxin acts by

forming lesions in the lipid bilayer of the host cell membrane. The cell then loses contents such as adenosine triphosphate (ATP), calcium ions and adenosine diphosphate (ADP), leading to cell death (Bhakdi *et al.*, 1991).

Exfoliative toxins are serine proteases that are able to break down specific proteins of the skin, known as desmosomal proteins. Therefore, skin layers become separated and appear as peeling, which is known as scalded skin syndrome (Nishifuji *et al.*, 2008).

Toxic shock syndrome toxin (TSST) is a superantigen which causes toxic shock syndrome (TSS) through non-specifically stimulating T- lymphocytes (Buonpane *et al.*, 2005). TSS is characterised by acute fever with rash, hypotension and multiple organs failure. TSST shows an ability to be absorbed *via* mucosa and it enters the bloodstream that leads to influx T lymphocytes to the site of infection (Buonpane *et al.*, 2005). T lymphocytes secrete large quantities of cytokines and can affect the circulatory system because the outcome of this excessive secretion causes capillary leakage that leads to serious hypotension and shock (McCormick *et al.*, 2001).

1.3.2.3 Panton-Valentine leukocidin

Uniquely, CA-MRSA contains *lukS* and *lukF* genes (Vandenesch *et al.*, 2003). These genes encode a specific bi-component leukocidin which is known as PVL. This toxin belongs to a β-barrel forming family of cytolytic toxins (Szmigielski *et al.*, 1999). The toxin is a major virulence factor in the pathogenesis of CA-MRSA, but studies have produced conflicting findings (Badiou *et al.*, 2008; Badiou *et al.*, 2010; Lipinska *et al.*, 2011; Panton *et al.*, 1932; Bubeck Wardenburg *et al.*, 2008; Kobayashi *et al.*, 2011; Voyich *et al.*, 2006).

PVL production is associated with skin infections in CA-MRSA disease (Badiou *et al.*, 2008). Several reports show a correlation between increased production of PVL and the formation of abscesses in the skin (Badiou *et al.*, 2008; Badiou *et al.*, 2010; Lipinska *et al.*, 2011; Panton *et al.*, 1932). The concentration of PVL was raised significantly in human clinical samples infected with PVL(+) strains of CA-MRSA (Badiou *et al.*, 2010). PVL had a significant impact on skin abscesses when compared to PVL(-) strains of CA-MRSA (Badiou *et al.*, 2008; Badiou *et al.*, 2010; Lipinska *et al.*, 2011; Panton *et al.*, 1932), whereas other researchers failed to detect this effect, showing no difference in abscess formation between PVL(+) strains and PVL(-) strains of CA-MRSA (Bubeck Wardenburg *et al.*, 2008; Kobayashi *et al.*, 2011; Voyich *et al.*, 2006). A proposed reason for this unexpected findings is the different growth media used in experiments (Graves *et al.*, 2010). This may explain why there are different findings from the studies of Badiou *et al.* (2008, 2010), Voyich *et al.* (2006) and Bubeck *et al.* (2008). Moreover, another potential explanation of these conflicting reports is the different mode of injection in the skin infection that was used in the studies of Lipinska *et al.* (2011) and Kobayashi *et al.* (2011).

The effect of PVL on neutrophils and its ability to lyse them has been debated (Hongo *et al*., 2009; Genestier *et al*., 2005; Löffler *et al.,* 2010). PVL has been reported to lyse human and rabbit neutrophils at concentrations of 0.3 to 2 μg/ml (Genestier *et al.*, 2005; Löffler *et al.*, 2010). While, PVL has a less cytolytic effect on murine neutrophils (Hongo *et al.*, 2009). Thus there is a species-specific interaction between PVL and host cells behind the difference reported by Badiou *et al.* (2008, 2010), Voyich *et al.* (2006) and Bubeck *et al.* (2008).

Another report shows that phenol-soluble modulins (PSMs) have an important role in CA-MRSA infection, in addition to PVL (Hongo *et al.*, 2009). Furthermore, the cytolytic effect of PVL against human and rabbit neutrophils is enhanced by phenol-soluble modulin α3 (PSMα3) (Hongo *et al.*, 2009).

1.3.2.4 Phenol-soluble modulin toxins

PSM toxins are small peptides produced by *S. aureus* (Wang *et al.*, 2007). PSM toxins are divided into two types, based on their length; α-PSMs and β-PSMs. The length of α-PSMs is ~20 amino acids, while length of β-PSMs is ~44 amino acids (Laabei *et al.*, 2014). These amino acid residues provide pro-inflammatory activities and α-helicity features to the PSM toxins. Lysine residues at positions 6 and 12 and an N-terminal formyl group are functional requirements for pro-inflammatory activities (Towle *et al.*, 2016). The PSMα toxins have a high degree of amphiphilicity with a helical shape. For example, the hydrophilic phase of the helical shape of PSMα3 has the largest number of negative and positive charges of amino acids connected by ionic bonds (Mehlin *et al.*, 1999).

The alpha-helicity is essential for the ability of PSMs to cooperate with lipid membranes which allows for the formation of both hydrophilic and hydrophobic phases of the helical shape of PSMs, leading to disruption of the cell membrane (Towle *et al.*, 2016). Each of the α-PSMs contains a single α-helix that runs from residue 2 to 19 or 20 with a slight bend at residue 6. The last helix of β-PSMs starts at residue 25 to 43. These helices are stabilised by salt bridges (Towle *et al.*, 2016). The salt bridges are in different positions according to the type of PSMs. For example, the location of salt bridges in PSMα1

appears between Lys12 and Glu16, while in PSMα3 in between Asp13 and Lys17 residue (Towle *et al.*, 2016).

S. aureus PSMα3 has a superior lytic activity over other classes of PSM toxins against neutrophils, principally because of its large hydrophobic moment. Therefore, it has a significant role in the pathogenesis of CA-MRSA (Wang *et al.*, 2007).

The cytolytic activity of PSMs occurs independently of its receptor, FPR2 (Kretschmer *et al.*, 2010). This PSM receptor mediates pro-inflammatory events that are not essential for cytolysis (Kretschmer *et al*., 2010). The observed cytolytic action of PSM on cells such as neutrophils and erythrocytes, occurs by disruption the integrity of their plasma membranes (Cheung *et al*., 2012; Wang *et al*., 2007). The cytolytic mechanism of PSMs starts with initial attachment and perturbation of the plasma membrane at micromolar concentrations of the PSM peptides. Then pores form in the plasma membrane leading to cell lysis. This mechanism is similar to that described for the cytolytic mechanism of δ-toxin (Talbot *et al.*, 2001). The cytolytic effects of PSMs are proposed to be due to their structure; α-helical with high degree of amphiphacity. These properties allow the peptides to integrate in the plasma membrane, thus forming pores which leads to the cell death (Wang *et al*., 2007).

Cytolytic effects of PSMs have been shown on variety of human cells such as neutrophils and erythrocytes (Cheung *et al*., 2012; Wang *et al*., 2007). The α-type PSMs have higher cytolytic capacities toward neutrophils than other types. For example, *S. aureus* PSMα3 has high cytolytic activity toward neutrophils that contributes to the pathogenesis of

infections of CA-MRSA (Wang *et al*., 2007). Furthermore, PSMs can lyse erythrocytes at micromolar concentrations (Cheung *et al*., 2012). However, the relevance of this cytolytic effects has been debated because of the presence of serum lipoproteins in the blood that neutralise PSMs (Surewaard *et al.*, 2012). As mentioned previously, this cytolytic action is receptor-independent and it occurs by disruption of the plasma membrane (Kretschmer *et al.*, 2010).

PSMs at nanomolar concentrations stimulate inflammatory responses, but at micromolar concentrations cause cell lysis (Wang *et al*., 2007). These peptides have an ability to attract neutrophils and stimulate them to release cytokines (Wang *et al*., 2007). In contrast to cytolysis, the pro-inflammatory activity of PSMs is mediated by an interaction with FPR2 (Kretschmer *et al*., 2010), because PSMs are secreted with N-formyl methionine that confer recognition by this receptor. Stimulation of FPR2 may be considered as part of the pathogenic process of *S. aureus* (Kretschmer *et al*., 2010). Activation of this receptor causes massive neutrophil influx that leads to tissue destruction (Kretschmer *et al*., 2010). FPR2 is present on other immune cell, such as dendritic cells (DCs). The importance of these cells is their link between the innate and adaptive part of immune system. PSMs cause release IL-10 from DCs that reduces secretion of other pro-inflammatory cytokines and enhances immune evasion of *S. aureus* (Schreiner *et al*., 2013). This action of PSMs on DCs may be an FPR2-independent process (Schreiner *et al*., 2013).

PSMs also have a role in the structuring of the *S. aureus* biofilms, via detaching bacterial cells, leaving gaps between them that give the biofilm its structure of cell towers or

mushroom-like shapes, formed with fluid-filled channels (Periasamy *et al.*, 2012). The role of the PSMs in this phase is to form pores in the matrix of biofilm due to a limitation of oxygen and nutrition, which leads to spread the bacteria to other new sites (Periasamy *et al.,* 2012).

Cheung *et al*. (2014) have studied the structure-function relationship of PSMα3, because this peptide has a strong cytolytic activity among the PSMs (Cheung *et al.*, 2014). This work used an alanine substitution screen to determine specific amino acid positions in PSMα3 that are responsible for its biological activities (Cheung *et al.*, 2014). These data showed that amino acids located on the hydrophobic and hydrophilic faces are responsible for the cytolytic function. K6, K12, K17 and L7 residues are responsible for the cytolytic activity (Cheung *et al.*, 2014). Exchange of amino acids with alanine leads to strongly decreased lysis of neutrophils and erythrocytes (Cheung *et al.*, 2014). Furthermore, residues responsible for pro-inflammatory activity are located on hydrophilic side of the PSMα3 peptide. K6, K12, K17, N21 and N22 residues of the peptide play role in activation of FPR2-mediated pro-inflammatory activity of PSMα3. Moreover, amino acids on the hydrophobic sides of peptide are responsible for structuring of biofilms (Cheung *et al.*, 2014).

1.3.2.4.2 Secretion of PSM toxins

PSM peptides are exported *via* a four-component ATP-binding cassette transporter (ABC transporter), which is known as phenol-soluble modulin transporter (Pmt) (Figure 1.2) (Chatterjee *et al.*, 2013). Pmt is a special system of ABC transporter that is responsible for exporting all types of PSM toxins to the outside environment. The locus of *pmt* contains four genes: *pmtA* and *pmtC* that encode ATPases, and *pmt*B and *pmt*D that encode membrane-associated proteins (Chatterjee *et al.*, 2013).

The mechanism of the Pmt system is not fully understood. However, there is a hypothetical mechanism which suggests that Pmt works *via* either a "vacuum cleaner" or flippase mechanism. It is dependent on whether the PSM molecule is secreted to the extracellular aqueous environment, or is just flipped to the outer membrane layer of the bacterial cell (Choudhury *et al.*, 2014). The hypothetical mechanism has the PSM molecule incorporated into the membrane and binds to the ligand-binding site of Pmt, which is found within the membrane and not the cytoplasm. Acceptance of PSM by the ligand-binding site occurs in the inward-facing open state of Pmt. This binding leads to stimulation of ATP binding by ATPases due to change in the outward occluded conformation. Finally, the PSM molecule is expelled to the extracellular aqueous environment (Choudhury *et al.*, 2014).

The presence of the Pmt transporter is essential for secreting all PSMs in *S. aureus* (Chatterjee *et al.*, 2013). A lack of Pmt leads to the accumulation of PSM toxins in the cytoplasm of *S. aureus* and consequently causes bacterial death (Chatterjee *et al.*, 2013). Homologues of Pmt genes are only found in staphylococcal species (Chatterjee *et al.*, 2013).

The presence of the Pmt transporter provides immunity for *S. aureus* when self-produced PSMs peptides are expressed (Chatterjee *et al.*, 2013). This transporter facilitates PSM secretion from the cytosol to the extracellular environment (Chatterjee *et al.,* 2013). This system is essential for bacterial survival and growth, as absence of Pmt causes accumulation of PSMs in the cytosol, leading to serious impairment of growth of the bacteria (Chatterjee *et al.,* 2013). Moreover, Pmt confers a protective immunity for *S. aureus* against PSMs that are produced by other staphylococcal species during the host co-colonisation. These PSMs may be produced by *S. epidermidis* that are co-colonised with human epithelia of skin (Cogen *et al.*, 2010). These findings demonstrated that Pmt confers immunity towards self and non-self PSMs that have a significant impact on the development of *S. aureus* infections (Chatterjee *et al.,* 2013).

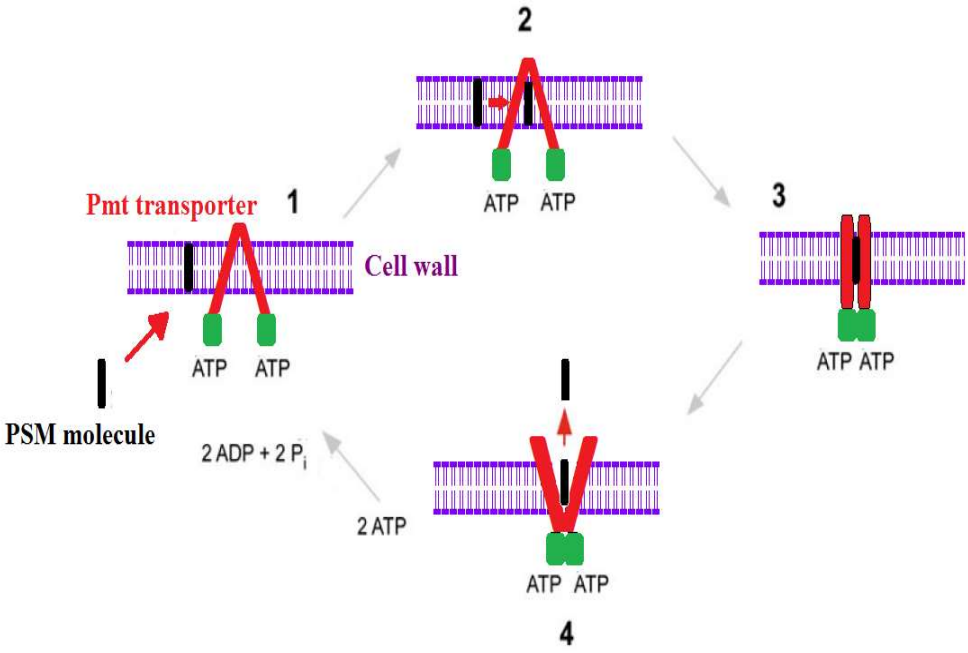

Figure 1.2: Exporting of PSM toxins. The Pmt transporter is responsible for exporting PSMs from the cytosol of *S. aureus* (Chatterjee *et al.*, 2013). There are four stages of the hypothetical vacuum cleaner mechanism of the Pmt system for exporting PSM peptides. **(1)** Integration of the PSM molecule into the membrane. **(2)** Binding of the PSM molecule to the ligand-binding site of the Pmt transporter. **(3)** Conformation of outward occlusion due to stimulation of ATP binding. **(4)** Release of the PSM molecule to the extracellular aqueous environment. Figure adapted from Dickey (2006).

1.3.2.4.3 Current opinions about role of PSM toxins

1.3.2.4.3.1 Impact of PSM toxins on skin and sepsis infections with CA-MRSA

As discussed earlier, PSMs play a central role in different facets of CA-MRSA pathogenesis, confirming that these peptides have an impact on the progression of CA-MRSA disease. The severity of diseases such as skin and soft tissue infections, and sepsis caused by CA-MRSA, correlates with the production of PSMs, because of their cytolytic and pro-inflammatory activities (Kretschmer *et al.*, 2010). Accordingly, studies that compare CA-MRSA strains which the wild-type strains and isogenic *psm*α mutant strains in models of infection (Cassat *et al.*, 2013; Cheung *et al.*, 2012; Giese *et al.*, 2011; Wang *et al.*, 2007). PSMs play an integral role in abscess formation in the skin. This infection represents around 90% of all clinical presentation of CA-MRSA disease (Deleo *et al.*, 2010). Virulence of CA-MRSA strains is attenuated in a model of skin infection when PSMs genes are deleted (Wang *et al.*, 2007). Deletion of the *psm*α causes loss of the ability of bacteria to form subcutaneous abscesses in the skin (Wang *et al.*, 2007). Therefore, deletion of the *psm*α specifically, causes decrease potency of bacteria towards formation of abscesses. Moreover**,** PSMs show an ability to elicit a cytotoxic effect on blood cells that allows to CA-MRSA infection to spread within the body of the host (Cassat *et al*., 2013; Cheung *et al*., 2012; Giese *et al*., 2011; Kretschmer *et al.*, 2010). To conclude, cytolytic and pro-inflammatory activities of PSMs have a significant contribution towards disease progression of CA-MRSA.

Moreover, several observations found PSMs to be a powerful chemoattractant for immune cells such as neutrophils (Kretschmer *et al.*, 2010; Rautenberg *et al.*, 2011) and dendritic cells (DCs) (Schreiner *et al.*, 2013). PSMs are able to activate these cells at

nanomolar concentrations (Kretschmer *et al.*, 2010; Rautenberg *et al.*, 2011). The cytolytic effect of PSMs is associated with severe infection of CA-MRSA. For example, the α-type PSM peptides lyse neutrophils at micromolar concentrations. This toxicity of PSMs is due to their high degree of amphiphacity and the α-helical structure (Towle *et al.*, 2016). The α-type PSMs destroy neutrophils, consequently damaging the immune system (Kretschmer *et al.*, 2010), although α-type PSMs have a non cytolytic effect on DCs at micromolar concentrations (Schreiner *et al.*, 2013). Cytotoxicity of PSMs is also observed with different host cells such as osteoblasts, epithelial cells and erythrocytes (Cassat *et al.*, 2013; Cheung *et al.*, 2012; Giese *et al.*, 2011). However, the ability of PSMs to elicit a cytotoxic effect on those cells is varied. Concentrations and time of exposure have a significant effect on PSMs activity. For example, the α-PSM peptides cause lysis of osteoblasts at concentrations up to 100 μg/mL, after 23 hours incubation. Horse erythrocytes become lysed after 45 min incubation with 62.5 μg/mL of α-PSM peptides. On the other hand, the β-PSM peptides have less cytolytic activity when compared to the α-PSMs (Cheung *et al.*, 2012; Wang *et al.*, 2007).

1.3.2.4.3.2 Detection of PSM toxins by host immune system

PSMs have an N-formyl methionine (Towle *et al.*, 2016) which is recognised as a signal for bacterial invasion by the host's immune system (Fu *et al.*, 2006). The interaction of neutrophils with PSMs was investigated by Kretschmer *et al.* (2010). This research group reported human formyl peptide receptor 2 (FPR2) as the main receptor for sensing PSMs (Kretschmer *et al.*, 2010). FPR2, also named formyl peptide receptor-like 1, belongs to the family of GPCRs and is found on immune cells such as neutrophils and monocytes (Migeotte *et al.*, 2006) and also found in megakaryocytes (Czapiga *et al.*, 2005). FPR2

exists with two paralogs which are formyl peptide receptor 1 (FPR1) and formyl peptide receptor 3 (FPR3) (Migeotte *et al.*, 2006).

Kretschmer *et al.* (2010) showed that PSMs at nanomolar concentrations are able to induce neutrophil chemotaxis *via* FPR2. The specificity of this interaction between FPR2 and PSMs has been analogue using the CHIPS (de Haas *et al.*, 2004) and FPR2 inhibitory protein (FLIPr) (Prat *et al.*, 2006). CHIPS and FLIPr are protein inhibitors that are produced by *S. aureus* which block the ability of FPR1 and FPR2 to detect formylated peptides , respectively. FLIPr reduces the ability of FPR2 to recognise PSMs (Kretschmer *et al.*, 2010).

Other researchers have different opinions about the recognition of PSMs by host cells, Hajjar *et al*. (2001) investigated the response of the Toll-like receptor 2 (TLR2) to PSM toxins that are produced from *S. epidermidis* and found that PSM toxins cause activation of TLR2 in human cells (Hajjar *et al.*, 2001). Moreover, Hanzelmann *et al*. (2016) reported that PSMs and lipoproteins together, obtained from staphylococcal culture supernatants, cause strong stimulation of TLR2 (Hanzelmann *et al.*, 2016).

Finally, Schreiner *et al*. reported that PSMs work independently of FPR2 in DCs and cause inhibition of DC functions (Schreiner *et al.*, 2013). Thus, PSMs show the ability to manipulate host cells through different ways that help *S. aureus* to establish an infection.

1.3.2.4.3.3 Regulation of PSMs

PSMs are regulated by the accessory gene regulator (*agr*) in a similar way to other staphylococcal virulence factors, described later in the section 1.5 (Wang *et al.*, 2007, Queck *et al.*, 2008). In common with many other *S. aureus* virulence factors, PSMs are regulated by the *agr* system via its regulatory RNAIII (Wang *et al.*, 2007; Queck *et al.*, 2008). Additionally, Queck *et al.* (2008) showed that AgrA binds promoters of *psm* genes, directly regulating their transcription.

Recent studies show that aureolysin, a protease controlled by *agr*, staphylococcal accessory regulator (SarA) and SaeRS, degrades PSMs and thus inactivate the toxins (Cassat *et al.,* 2013; Rasigade *et al.*, 2013; Zielinska *et al.*, 2011). SarA does not control levels of PSM gene transcription, but works through decrease levels of PSMs degradation by down-regulating of aureolysin expression (Zielinska *et al.,* 2011). SaeRS inactivation causes increased levels of aureolysin that leads decreases levels of PSMs (Cassat *et al.,* 2013).

Interestingly, expression of PSMs is activated in intracellular environments (Geiger *et al.*, 2012; Surewaard *et al.,* 2012). When *S. aureus* is phagocytosed by neutrophils, it leads to activation of the stringent response. The bacterium rapidly secretes (p)ppGpp, an intracellular signaling molecule, that leads to immediate activation of PSM production through an undetermined mechanism (Geiger *et al.,* 2012). Thus, the production of these toxins enhances the ability of bacteria to elicit phagosomal escape.

1.4 *S. aureus* immune evasion

S. aureus can escape elements of innate immune system namely antimicrobial peptides, innate leukocytes and complement to successfully colonise the host (Medzhitov *et al.*, 2000). The complement system is composed of a series of circulating proteins that become activated in order to detect invading microbes within the bloodstream (Dunkelberger *et al.*, 2010). Phagocytes such as neutrophils and macrophages have the ability to destroy invading microbes through producing substances such as reactive oxygen species, lysozyme and antimicrobial peptides (Foster, 2005).

1.4.1 Escaping antimicrobial molecules

Antimicrobial molecules, such as thrombocidins and cathelicidins, are found in high concentrations in platelets and neutrophils (Peschel *et al.*, 2001). These molecules are positively charged and are called cationic antimicrobial peptides (CAMPs) (Peschel *et al.*, 2001). They bind to the surface of *S. aureus* due to the cell's net negative charge (Kraus *et al.*, 2008). *S. aureus* can resist CAMPs by cleaving or chelating them and thus leads to these CAMPs being inactivated (Kraus *et al.*, 2008; Peschel, 2002). For example, cathelicidin LL-37 becomes inactivated through cleaving by aureolysin and serine-protease V8 that are secreted by *S. aureus* (Sieprawska-Lupa *et al.*, 2004). This bacterium is also able to modify its charge by introducing D-alanine into teichoic acids and therefore providing a positive charge to the structure of peptidoglycan (Collins *et al.*, 2002).

1.4.2 Escaping phagocytes

S. aureus can escape phagocytes by inhibiting their chemotaxis (Haas *et al.*, 2005), resisting their respiratory burst (Clauditz *et al.*, 2006) and by formation of pores in their

membranes (Yoong *et al.*, 2013). *S. aureus* produces a small protein called chemotaxis-inhibitor protein of *S. aureus* (CHIPS). CHIPS blocks C5a and FPR1 receptors on neutrophils from recognising these ligands (de Haas *et al.*, 2004; Postma *et al.*, 2004).

Moreover, staphylokinase, an enzyme produced by *S. aureus*, shows a role in survival of the bacterium within the bloodstream *via* formation of plasmin, by cleaving plasminogen (Rooijakkers, van Wamel*, et al.*, 2005). The bacterium uses this mechanism to hide itself inside a fibrin clot, escaping from immune recognition (Rooijakkers, van Wamel*, et al.*, 2005).

S. aureus shows an ability to resist the respiratory burst of neutrophils that includes reactive oxygen species that are produced in the phagolysosome (Robinson, 2008). Reactive oxygen species cause oxidation of bacterial surface components. *S. aureus* produces staphyloxanthin which is a carotenoid pigment that has antioxidant activity through inactivation of free radicals (Clauditz *et al.*, 2006).

1.4.3 Escaping complement system

S. aureus can impair direct lysis and prevent opsonisation by the complement system (Rooijakkers, van Kessel*, et al.*, 2005). Staphylokinase causes disruption of the complement cascade *via* activated plasmin that cleaves IgG and C3b bound to the surface of the pathogen (Rooijakkers, van Kessel*, et al.*, 2005). *S. aureus* surface protein (SpA) interacts with surface-bound IgG *via* binding to its Fc region. This region is recognised by C1q which is required for activation the classical pathway of the complement system (Atkins *et al.*, 2008). This inhibition leads to prevention of activation of the complement system (Atkins *et al.*, 2008).

1.5 Regulation of virulence factors of *S. aureus*

The accessory gene regulator (*agr*) is a regulator for virulence factors of *S. aureus*. *agr* coordinates expression of many genes of *S. aureus* that are involved in adhesion, cell metabolism and toxin production (Recsei *et al.*, 1986). The *agr* locus is composed of two operons which are controlled by two divergent promoters, P2 and P3.

The promoter P2 initiates RNAII transcription which is responsible for density-dependent signaling. The promoter P3 initiates the transcription of RNAIII, which is the effector RNA. Transcription of RNAII and RNAIII leads to stimulation or repression other genes (Janzon *et al.*, 1989; Novick *et al.*, 1995). *agrBDCA* are encoded on the RNAII transcript. These genes have functions in signal generation, release, and detection.

The AgrD propeptide is processed by AgrB, a transmembrane peptidase, which secretes small auto inducing peptides (AIPs) (Figure 1.3) (Qiu *et al.*, 2005; Saenz *et al.*, 2000). The AIPs bind AgrC, which is a transmembrane histidine kinase receptor, leading to phosphorylation of AgrA. AgrA has an affinity for the P2 promoter that leads to immediately stimulating it (Koenig *et al.*, 2004; Lina *et al.*, 1998). Therefore, a positive feedback loop comes into play through AIP accumulation that leads to more production of AIP. The P3 becomes activated when an activation threshold is reached. RNAIII is responsible for both the increased expression of exotoxin genes and decreasing expression of surface proteins (Rutherford *et al.*, 2012).

Expression of *agr* has been studied under *in vitro* and *in vivo* conditions. Secretion and accumulation of AIP are required for upregulation of *agr*. Simultaneous release of AIP

occurs during the transition from exponential to stationary phase in *vitro* conditions (Ji *et al.*, 1995).

agr plays an important role in the pathogenesis of bacteria. *agr* mutants showed a decreased ability to cause infections such as abscesses, osteomyelitis, septic arthritis and endocarditis (Abdelnour *et al.*, 1993; Cheung *et al.*, 1994; Cheung *et al.*, 2011; Gillaspy *et al.*, 1995). This observation was due to reduced or absent production of exotoxin and surface proteins (Recsei *et al.*, 1986).

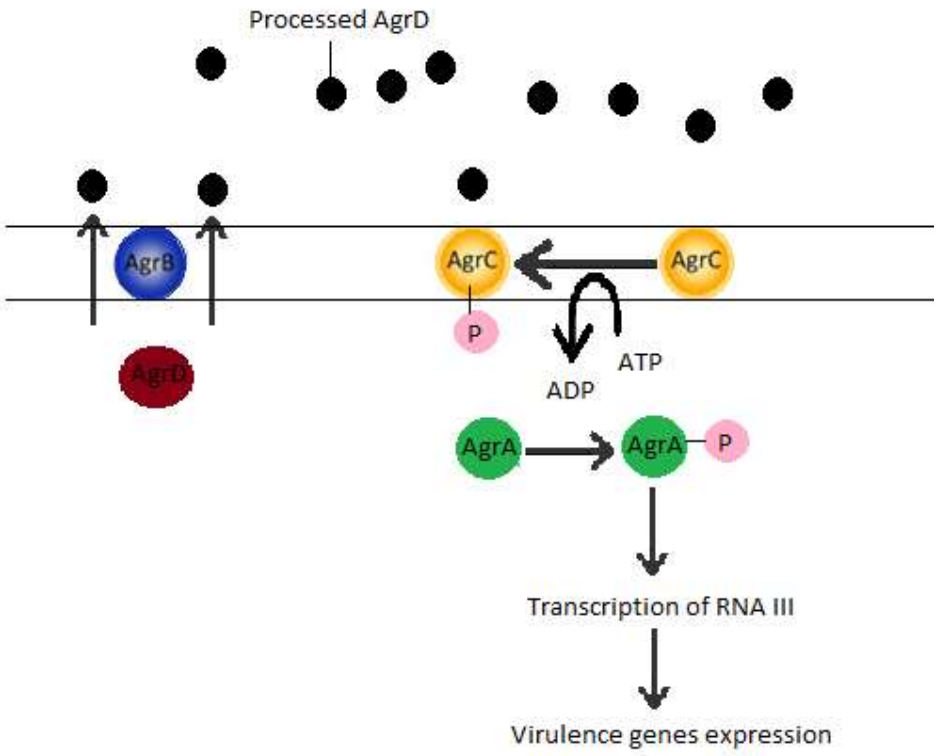

Figure 1.3: The Agr system regulates transcription of virulence genes. AgrB processes AgrD. High concentrations of AgrD cause phosphorylation of AgrC. This leads to phosphorylation of AgrA that causes transcription of RNA III which leads to expression of virulence genes (Cheung *et al.*, 2014).

1.6 Platelet formation and clearance

Human platelets are small anucleated blood elements, with a size of 2.0-5.0 μm in diameter and are derived from megakaryocytes (Deutsch *et al.*, 2006). The thickness of a platelet is approximately 0.5 μm, and it has a life span of 7-10 days. The average normal count of human platelets is 150,000-400,000/μl of blood (Nieswandt *et al.*, 2011). Their main function is to maintain haemostasis, but they are also involved with the innate immune defences of the host (Cox *et al.*, 2011).

Platelet formation occurs in the bone marrow through a process known as haematopoiesis (Deutsch *et al.*, 2006). Proplatelets are developed from mature megakaryocytes (Deutsch *et al.*, 2006). These proplatelets have long thin cytoplasmic extensions that have microtubules that play a role in elongation of these cells that eventually form platelets. Moreover, the force of actin also plays an important role in forming platelets from proplatelets, through "pinching off" (Patel *et al.*, 2005).

Newly formed platelets circulate within the blood (Kaplan *et al.*, 1978) and are glycosylated on their platelet receptors such as glycoprotein VI (GPVI) and GPIb-V-IX with a sialic acid cap that protects them from clearance (Crook, 1991). When platelets age, the sialic acid cap is removed and becomes exposed to ashwell-Morell receptors (AMR) on hepatocytes (Grozovsky *et al.*, 2015). This detection of desialylated platelets by AMR leads to clearance of old platelets by the liver (Kaplan *et al.*, 1978).

1.7 Platelet structure

The structure of platelets (Figure 1.4) can be divided into different zones; peripheral, the sol–gel and the organelle zones (White, 2004). The peripheral zone is composed of

platelet membrane with surface embedded receptors and the open canalicular system (OCS), which is a channel that connects the external surface to the cytoplasm. The sol–gel zone is a transparent matrix formed by a meshwork of fibrous that is required for embedding organelles. The organelle zones are composed of α granules, dense granules and lysosomes (White, 2004). The α-granules are unique and the most abundant granules in the platelet with a diameter of 200-500 nm (Stadelmann *et al.*, 1998).

The α granules contain P-selectin which belongs to the selectin family. P-selectin is also found in the Weibel-Palade bodies in endothelial cells. During the activation of platelets, the stored P-selectin is transported to the surface of the activated platelet, which is important for the recruitment of other platelets through its lectin domain that binds to nearby lectin domains of other activated platelets. Exposure of P-selectin plays a role in platelet-leukocyte interactions. This interaction occurs through engagement of P-selectin with glycoprotein ligand 1 (PSGL-1) on the surface of leukocytes. PSGL-1 is considered as a primary ligand for P-selectin (McEver *et al.*, 1997). Binding of P-selectin to PSGL-1 leads to the tether and roll on leukocytes to endothelial cells at the site of injury (McEver *et al.,* 1997; Tedder *et al*., 1995).

Dense granules have a diameter of approximately 150 nm and look like dense bodies under electron microscopy (White, 2004). This is due to high amounts of Ca^{2+} and phosphate that are stored in these granules (Fukami, 1992). The last type of granule is the lysosome that degrades the protein and carbohydrate. Lysosomes have lysosomal-associated membrane proteins such as LAMP-1, LAMP-2 and LAMP-3 (Saftig *et al.*, 2009).

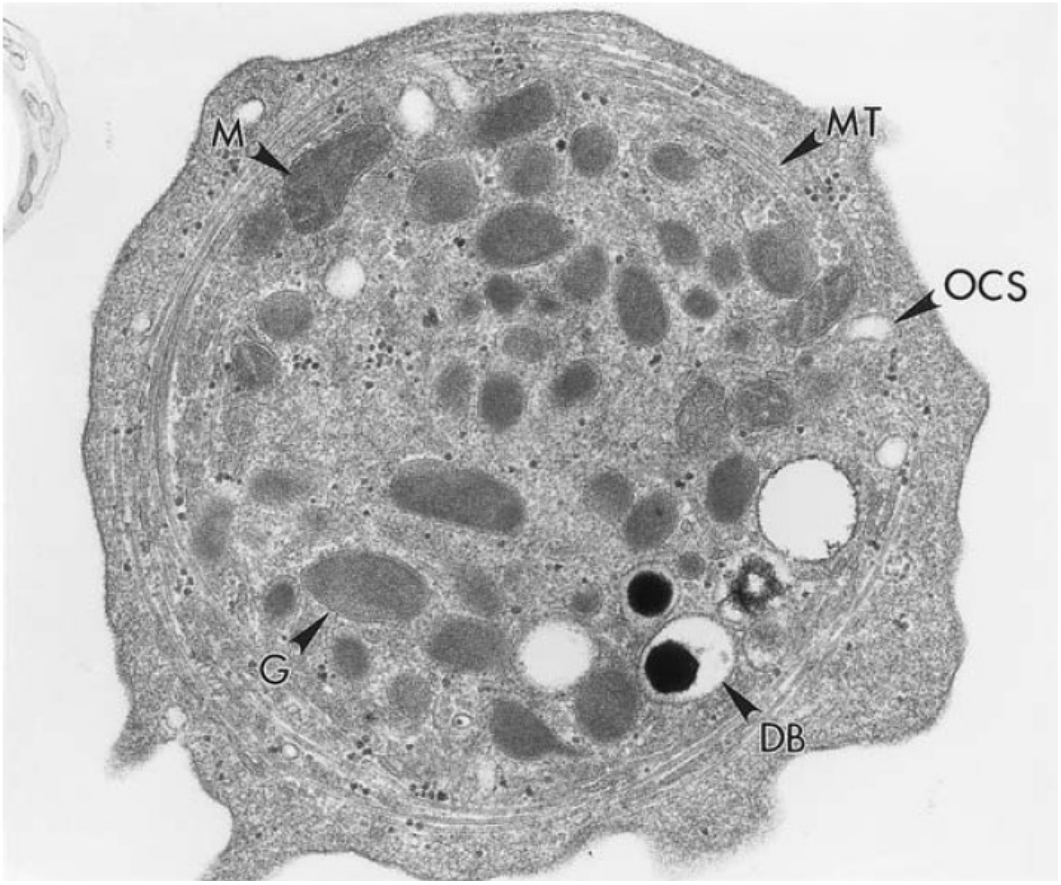

Figure 1.4: Structure of a human platelet. Ultrastructure of platelet with a discoid shape. The image shows: circumferential coil of microtubules (MT), dense bodies (DB), open canalicular system (OCS), alpha granules (G) and mitochondria (M) (White, 2004).

1.8 Platelet functions

1.8.1 Haemostasis

Haemostasis is the main function of platelets, preventing blood loss at the site of an injury. In flowing blood, large blood cells such as red blood cells push platelets to circulate near the wall of blood vessels (Aarts *et al.*, 1988). This leads to a rise in the concentration of platelets at the wall of blood vessels.

Under normal conditions, platelets circulate in a resting phase within the cardiovascular system. Upon injury of the blood vessel wall, the subendothelial matrix becomes exposed and releases molecules such as collagen and von Willebrand factor (vWF) which leads to activation of platelets (Furie *et al.*, 2005). Consequently, platelets release their contents to activate other nearby platelets that leads to them being trapped in the thrombus. Therefore, forming a plug at the injury site (Anitua *et al.*, 2004).

1.8.2 Wound healing

The process of wound healing occurs through four main phases, which are haemostasis, inflammatory, proliferative and remodelling (Stadelmann *et al.*, 1998). Haemostasis is carried out by platelets and the coagulation system through formation of a platelet plug (Stadelmann *et al.*, 1998). Phagocytes are responsible for removing any debris from the wound site, including bacteria. The proliferative phase is necessary for reconstruction of the wound site by fibroblasts and this phase includes epithelialisation, deposition of collagen and angiogenesis (Barrientos *et al.*, 2008).

Platelets play a role in this phase and encourage migration of fibroblasts through secretion of the contents from their granules, such as serotonin, prostaglandins and bradykinin (Stadelmann *et al.*, 1998). Platelets also release vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) from their α granules, which can stimulate wound repair and vasculogenesis (Barrientos *et al.*, 2008). Therefore, new tissue is formed and the wound site is sealed permanently (Singer *et al.*, 1999).

1.8.3 Immune defence

Platelets are considered unique blood elements due to their function in innate immunity, along with their primary function in haemostasis (Cox *et al.*, 2011). The immune responses of platelets are classified into; recognition of invading pathogens, attacking pathogens through antimicrobial peptides (AMPs) and increasing recruitment of other immune cells of the host to the site of infection (Figure 1.5) (Scheuerer *et al.*, 2000). Recognition of invading pathogens by platelets is facilitated by complex interactions between these pathogens and platelet receptors. For example, platelets express Toll-like receptor 2 (TLR2), allowing them to detect cytomegalovirus (CMV) (Assinger *et al.*, 2014). This interaction of TLR2 with CMV leads to platelet activation (Assinger *et al.,* 2014).

The ability of platelets to attack pathogens is mediated by AMPs and modulation of inflammation. Thymosin β-4 (Tβ-4) and RANTES are AMPs that are produced by platelets and they have strong effects on some bacterial and yeast species (Tang *et al.*, 2002). Platelets play a role in the recruitment of immune cells to the site of an infection. For instance, α granules secrete platelet factor 4 (PF-4) which can activate neutrophils and recruit them to the site of an infection (Scheuerer *et al.*, 2000).

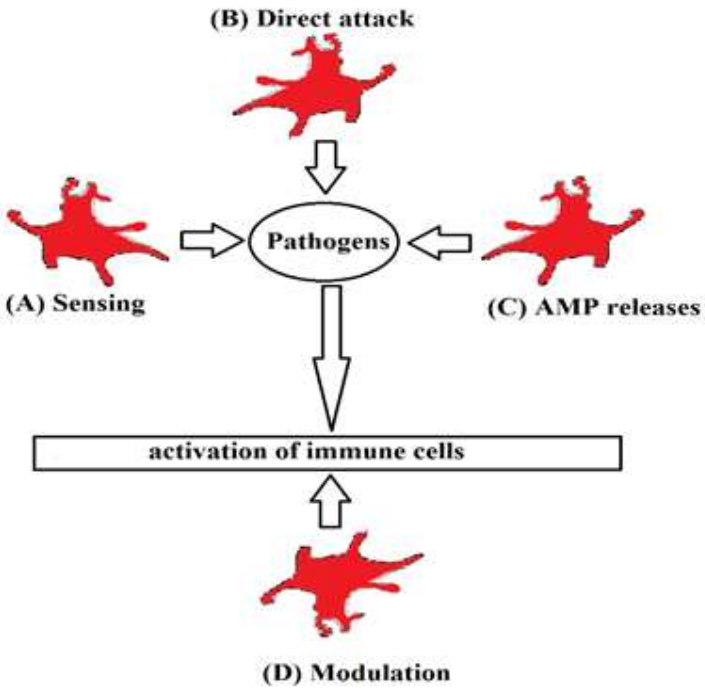

Figure 1.5: Immune functions of platelets. Platelets (in red) function to sense pathogens and attack them. Platelets interact with other immune cells. Diagram is adapted from Speth *et al.,* (2013).

1.8.4 Phagocytosis

Platelets share three common phagocytic properties with leukocytes; interaction with pathogens, possession of lysosomes in their cytoplasm and storage of inflammatory mediators (Meseguer *et al.*, 2002; Nachman *et al.*, 1972). Platelets can engulf small particles (<0.1 μm) (Lewis *et al.*, 1976). However, the ability of platelets to uptake bacteria in a manner similar to ingestion by neutrophils was a subject of controversy. Youssefian et al. (2002) showed that platelets engulf *S. aureus* in a similar way to phagocytosis by neutrophils. Although, they did not indicate if the *S. aureus* was killed after engulfment. This finding was a subject of controversy by White (2005). He postulated that platelets are just covercytes which only cover bacteria, because the size of bacteria is much greater than platelets. Therefore, they cannot be completely enclosed within platelets (White, 2005).

1.9 Platelet activation

1.9.1 Initial adhesion of platelets to the area of injury

Platelets turned into the activated form during vascular injury due to interaction of their receptors with ligands (Figure 1.6). Injured subendothelial components are sensed by platelet receptors like the GPIb-V-IX complex. Interaction of this receptor with vWF leads to the slowing down of circulating platelets and their rolling at the injured area. Eventually, collagen interacts with integrin $\alpha_2\beta_1$ that leads to initial weak adhesion at the site of injury (Watson *et al.*, 1998). Concomitantly, collagen interacts with GPVI-FcRγ that leads to clustering of this receptor (Jiang *et al.*, 2015). This causes FcRγ to phosphorylate which leads to the activation of the Src family kinases (Watson *et al.*, 1998). Eventually, a strong activating signal such as phospholipase C gamma (PLCγ) is

triggered (Pasquet *et al.*, 1999) which causes Ca^{2+} mobilisation and activation of integrin $\alpha_{IIb}\beta_3$ (Liu *et al.*, 2005). PLCγ is an enzyme that works through hydrolysing polyphosphoinositide (PI) 4,5P2 in the plasma membrane of platelets to form diacylglycerol (DAG) and inositol trisphosphate (IP3) (Jackson *et al.*, 1994).

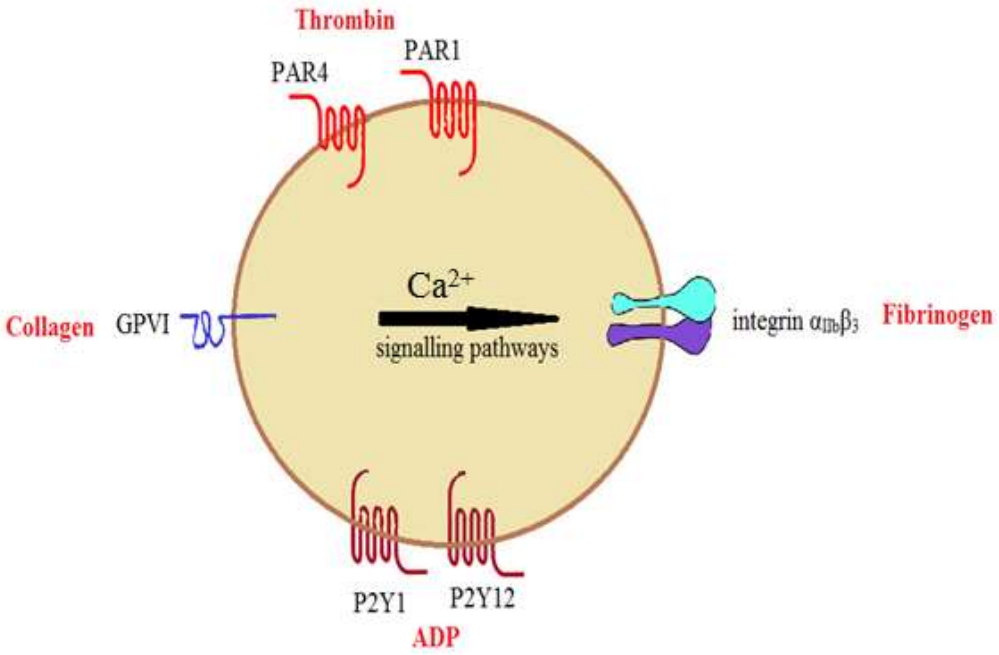

Figure 1.6: Major platelet receptors and their ligands. Binding of thrombin to PAR1 and PAR4 receptors. Binding of collagen to its main receptor GPVI. P2Y1 and P2Y12 are receptors for ADP. During platelet activation, a conformational shift from low state affinity to high affinity state of integrin $\alpha_{IIb}\beta_3$ to fibrinogen. It causes platelet adhere to other nearby platelet and aggregates at the site of injury. Figure adapted from Rivera *et al.,* (2009).

1.9.1 Ca^{2+} mobilisation

Ca^{2+} is stored in the dense tubular system (DTS) of human platelets and has an important role in their activation *via* rearrangement of the actin cytoskeleton, granule secretion and inside-out activation of the integrin $\alpha_{IIb}\beta_3$ (Soboloff *et al.*, 2007).

During platelet activation, IP3 causes release of the stored Ca^{2+} from the DTS into the cytoplasm of the platelet (Sage *et al.*, 1987). The level of intracellular Ca^{2+} rises to micro-molar levels and is followed by an influx of Ca^{2+} from the external medium. This occurs through the opening of the Ca^{2+} channels into the platelet cytosol to reach around 10 μM of intracellular Ca^{2+} in the plasma membrane (Sage *et al.*, 1987).

1.9.2 Platelet shape change

Upon activation of integrin $\alpha_{IIb}\beta_3$, the shape of the platelet changes from discoid to spherical shape with filopodia like extensions (Figure 1.7) (White, 2004). These cellular extensions appear due to dynamic rearrangement of the platelet cytoskeleton. This cytoskeletal rearrangement occurs due to depolymerisation of existing filamentous actin (F-actin) and synthesis of new F-actin (Brass, 2009).

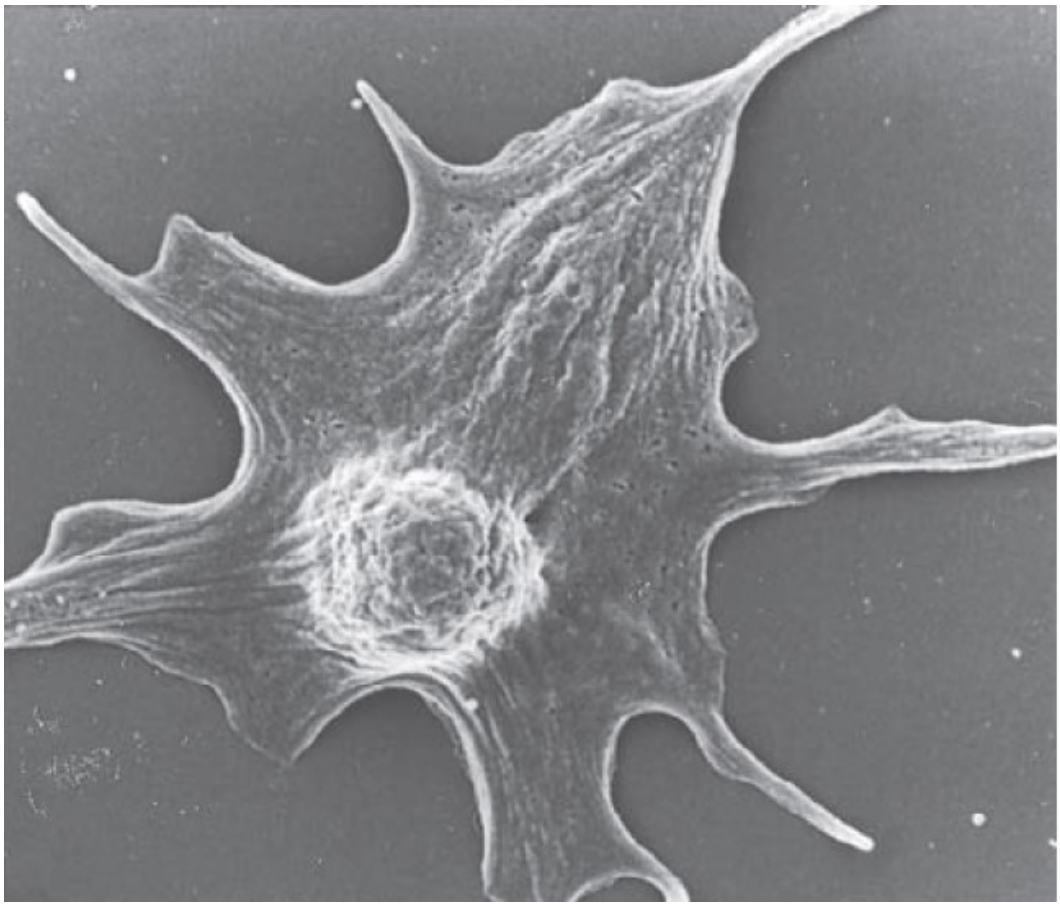

Figure 1.7: An activated platelet. An activated platelet with filopodia taken by White (2004).

1.9.3 Supporting platelet aggregation

Activation of integrin $\alpha_{IIb}\beta_3$ is necessary for platelet aggregation. It is composed of two transmembrane heterodimers; the α- and β-subunits. The interaction between these subunits leads to form the integrin $\alpha_{IIb}\beta_3$ (Wagner *et al.*, 1996).

A conformational change in integrin $\alpha_{IIb}\beta_3$ leads to binding soluble plasma fibrinogen (Ginsberg *et al.*, 1992). Fibrinogen is the primary ligand for integrin $\alpha_{IIb}\beta_3$ (Ginsberg *et al.*, 1992). The fibrinogen molecule is composed of two outer D domains and a central E domain. A coiled-coil segment links these domains. The molecule contains two sets of three polypeptide chains, which are Aα, Bβ and γ. These chains are connected together by five symmetrical disulfide bridges in the N-terminal E domain (Blombäck *et al.*, 1976; Henschen *et al.*, 1983; Hoeprich *et al.*, 1983; Huang *et al.*, 1993; Zhang *et al.*, 1992). Fibrinogen is a bipolar molecule, so can bind to $\alpha_{IIb}\beta_3$ integrin on two platelets, causing cross-linking of adjacent activated platelets (Leung *et al.*, 1986; Zucker *et al.*, 1985). This leads to the capture of other circulating platelets to the site of injury (Bennett, 2001).

1.9.4 Platelet spreading

Activated integrin $\alpha_{IIb}\beta_3$ triggers bi-directional signalling, known as inside-out and outside-in, across the platelet membrane (Shattil *et al.*, 1998). Changes in platelet shape from discoid to finger-like structures, followed by platelets being completely flattened out, leads to a fried egg like shape due to migration of organelles to the centre spreading the platelet over the injury site (Dooyoung Lee *et al.*, 2012).

1.9.5 Releasing of platelet granules' constituents

Platelet granules centralise and undergo homotypic fusion before they are secreted during platelet activation (Reed *et al.*, 2000). This process starts with the fusion of granules and the OCS (Flaumenhaft, 2003). The OCS works as a network for these fused granules and their membranes fuse with the plasma membrane of the platelet (Escolar *et al.*, 1991).

The α granules release coagulation factors like factor V and VIII that support clot formation (Heemskerk *et al.*, 2002). Dense granules release ADP that leads to maximal platelet activation. ADP binds to P_2Y_1 and P_2Y_{12} receptors on adjacent platelets that serves to recruit more platelets to the growing thrombus (Jin *et al.*, 2002).

1.9.6 Thromboxane A_2 generation

In activated platelets, thromboxane A_2 (TXA_2) is generated from arachidonic acid and binds to TP_α and TP_β receptors on other circulating platelets and subsequently activates them (Smyth, 2010). This process assists in the capture of more platelets in the growing thrombus (Dangelmaier *et al.*, 2001).

1.9.7 Thrombin generation

Thrombin is a potent inducer of platelet activation *via* cleavage of protease-activated receptor 1 (PAR1) and protease-activated receptor 4 (PAR4) in human platelets (Kahn *et al.*, 1999). It cleaves protease-activated receptor 3 (PAR3) in murine platelets (Nakanishi-Matsui *et al.*, 2000).

Thrombin also plays a role in formation of the fibrin through converting fibrinogen to fibrin (Wolberg *et al.*, 2008). This fibrin mesh makes the primary platelet plug more resistant to shear forces of flowing blood (Wolberg *et al.*, 2008).

1.9.8 Stabilisation of the clot

Consolidation of the clot is regulated by integrin $\alpha_{IIb}\beta_3$ outside-in signalling that transmits the retractile forces to the clot (Schoenwaelder *et al.*, 2010) and then the injured site in the blood vessel becomes closed (Anitua *et al.*, 2004). Upon this process, platelets achieve their main function which is stopping bleeding.

Clot retraction is the last event in the process of haemostasis in blood vessels. This process is mediated by $\alpha_{IIb}\beta_3$ and occurs after clot formation, *via* contractile forces which are exerted by the actin-myosin cytoskeleton (Schoenwaelder *et al.*, 2010). The actin-myosin cytoskeleton interacts with the internal domain of $\alpha_{IIb}\beta_3$. Clot retraction is important for assisting wound repair, preventing blockage of the blood vessel at the damaged site and managing thrombus stability (Schoenwaelder *et al.*, 2010).

1.10 Platelet inhibition

1.10.1 Nitric oxide and prostaglandin I_2

Nitric oxide (NO) and prostaglandin I_2 (PGI_2) are produced by the intact endothelium of blood vessels. NO and PGI_2 are considered as platelet ligands due to their ability to bind to them (Mitchell *et al.*, 2008). NO and PGI_2 modulate platelets, inhibiting their activation and maintaining them in a resting state (Mitchell *et al.*, 2008). The NO acts on platelets through binding to soluble guanyle cyclase (sGC) (Gkaliagkousi *et al.*, 2007).

The sGC leads to increased levels of cyclic guanosine monophosphate (cGMP) (Gkaliagkousi *et al.*, 2007).

PGI_2 acts on platelets by increasing the levels of cyclic adenosine monophosphate (cAMP) which leads to activation of protein kinase A (PKA). The process occurs due to binding of PGI_2 to prostacyclin receptor (IP) on the platelet surface (Raslan *et al.*, 2015).

1.10.2 Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor 1

Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor 1 (VPAC1) is found on the cell-surface membrane of platelets (Ishihara *et al.*, 1992). It consists of a C-terminal cytoplasmic tail, seven transmembrane domains and a long chain N-terminal extracellular end (Laburthe *et al.*, 1994; Ulrich *et al.*, 1998). The molecular weight of this receptor is approximately 52 kDa and it is composed of 457 amino acids (Goetzl *et al.*, 1995). VPAC1 receptor is expressed by megakaryocytes and platelets (Freson *et al.*, 2004).

VPAC1 receptor is now recognised as a negative regulator of platelet activation (Freson *et al.*, 2004; Freson *et al.*, 2008). Activation of VPAC1 receptor leads to reduced activation and aggregation of platelets (Freson *et al.*, 2004). The VPAC1 receptor is coupled to G proteins (Gs)-GPCR (Vaudry *et al.*, 2000), which increase the level of cAMP through activation of adenylyl cyclase (AC). AC stimulation leads to an increase in the cAMP levels by converting ATP to cAMP.

1.10.2.1 Abundance and natural ligands of VPAC1

VPAC1 receptor is abundant in several types of cells and tissues such as immune cells, the brain and layers of skin (Vaudry *et al.*, 2000). The receptor is most abundant in dendritic cells, macrophages, lymphocytes and mast cells (Delgado *et al.*, 2004), and is also expressed in the epidermis and dermis of the skin (Lundeberg *et al.*, 1999).

Vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are ligands of VPAC1 receptor (Harmar *et al.*, 2012). VIP and PACAP are neuropeptides that play roles in human physiology, such as control of the motility in the human intestine and secretion and regulation in the immune system (Moody *et al.*, 2011). VIP is a 28-amino acid peptide that was discovered in 1970 by Said and Mutt (Said *et al.*, 1970). PACAP is a 38-amino acid peptide that was first isolated from ovine hypothalamus (Miyata *et al.*, 1989). The two neuropeptides show ability to stimulate production of cyclic AMP in cells *via* binding to VPAC1 receptor (Harmar *et al.,* 2012). Interaction of VPAC1 receptor with its natural ligands occurred through binding to its N-terminal domain (Lins *et al.*, 2001).

1.10.3 Protein kinase A

The structure of PKA consists of two inactive catalytic subunits (C) and two regulatory subunits (R). These subunits of PKA become activated through binding of the regulatory subunits (R) by four cAMP molecules. This binding leads to the release of the catalytic subunits (C) to activate PKA (Potter *et al.*, 1979). The negative regulation of cAMP synthesis in platelets occurs via phosphodiesterases (PDEs) (Omori *et al.*, 2007) that manage the levels of cAMP inside the platelets.

1.11 Bacterial interaction with platelets

1.11.1 Interaction of *S. aureus* with platelets

The ability of surface proteins and toxins of *S. aureus* to interact with human platelets by binding to their receptors and affecting their normal physiological functions was reported (Fitzgerald *et al.*, 2006). The interaction of *S. aureus* with platelets can occur *via* one of two different pathways, either by binding to specific platelet receptors or by MSCRAMMs that are covered with plasma proteins (Loughman *et al.*, 2005). *S. aureus* also produces extracellular molecules that can activate or inhibit platelets.

1.11.1.1 *S. aureus* causes activation of platelets

ClfA and FnBPA can rapidly activate platelets *via* bridging of the fibronectin and fibrinogen that bind to platelet integrin $\alpha_{IIb}\beta_3$ (Figure 1.8). FnBPA can also activate platelets through binding to platelet Fc receptor FcγRIIa (Fitzgerald *et al.*, 2006). Binding of fibrinogen and fibronectin is important for *S. aureus* colonisation and invasion in damaged tissues. SpA can indirectly activate platelets via binding to regions in helices I and II in vWF (Hartleib *et al.*, 2000).

α-toxin can induce platelet aggregation at 1 nM (Bhakdi *et al.*, 1988) through releasing factor five (FV) from platelet α granules. Then, FV converts to factor five (a) (FV(a)) that assists to produce a prothrombinase complex on the platelet surface (Hemker *et al.*, 1967). This complex converts prothrombin to thrombin that leads to platelet activation (Hemker *et al.*, 1967).

Furthermore, α-toxin has an alternative way to trigger platelet aggregation through forming lesions in their surface that leads to an influx of Ca^{2+} ions into the platelet cytosol, which then leads to them becoming activated (Arvand *et al.*, 1990). Thus, activation of platelets can assist *S. aureus* to cause life threatening diseases such as IE (Sullam *et al.*, 1996).

The toxin induces platelets to synthesize B-cell lymphoma (Bcl-3) protein (Schubert *et al.*, 2011). Bcl-3 is present in activated platelets, but not in unstimulated platelets (Weyrich *et al.*, 1998). For example, Bcl-3 is found in platelets that are stimulated by collagen or thrombin (Pabla *et al.*, 1999; Weyrich *et al.,* 1998). Upon platelet activation, they translate mRNA for Bcl-3 into protein through signalling pathway includes phosphorylation of the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Upon synthesis of Bcl-3 in activated platelets, it binds to Fyn and regulates cytoskeletal changes that required for clot retraction (Weyrich *et al.*, 1998; Weyrich *et al.*, 2007). These similar effects of α-toxin and physiological platelet agonists. Thus, α-toxin can add to repertoire of platelet agonists that trigger Bcl-3 synthesis. Finally, rapid and prolonged activation of platelets in response to α-toxin has adverse consequences that play role in development of IE.

Binding of α-toxin to host cells requires its receptor, ADAM10, to cause cellular injury (Menestrina *et al.*, 2001; Wilke *et al.*, 2010). ADAM10 is abundantly expressed on human tissues and cells (Chantry *et al.*, 1990). This receptor is involved in regulation of tissue barrier and cellular activation (Reiss *et al.*, 2009). Interaction of the α-toxin with ADAM10 leads to activation of this receptor, resulting in cleavage of its substrates, such as receptors on target cells (Reiss *et al.,* 2009). GPVI receptor is considered as a major ADAM10 substrate (Bender *et al.*, 2010) and is the main platelet receptor for collagen (Jiang *et al.*, 2015). This receptor mediates activation of platelets in response to collagen found on exposed endothelium at the site of injury (Gibbins *et al.*, 1997). ADAM10 activation triggers GPVI proteolysis through cleavage of the ectodomain of GPVI (Bender *et al*., 2010; Powers *et al.*, 2015). α-toxin -ADAM10 complex mediates GPVI proteolysis leading to reduced platelet binding to collagen and thus impaired endothelial repair (Powers *et al*., 2015). Moreover, proteolysis of GPVI by the α-toxin-ADAM10 complex blunts activation of $\alpha_{IIb}\beta_3$ that is essential for aggregation (Powers *et al*., 2015). Thus, platelets fail to launch fibrinogen bridges between them, resulting in failure to aggregates and disability to form a plug at the injured endothelium (Tronik-Le Roux *et al.*, 2000).

S. aureus expresses iron-regulated surface determinant B (IsdB) that has a role in promoting invasion of bacteria into host cells (Zapotoczna *et al.*, 2013). IsdB is involved in interaction with platelets where it promotes the adhesion of bacteria to platelets (Miajlovic *et al.*, 2010). IsdB can induce aggregation through direct binding to $\alpha_{IIb}\beta_3$ (Miajlovic *et al.,* 2010). *S. aureus* also expresses Eap and serine-rich adhesin for platelets (SraP) involved in the interaction with platelets. These bacterial proteins allow adhesion to platelets (Siboo *et al.*, 2005). Eap can induce platelet activation and aggregation

through direct binding to glycosaminoglycans on the surface of platelets (Bertling *et al.,* 2012). SraP mediates the binding of *S. aureus* to platelets through mechanism that look like a receptor-ligand interaction (Siboo *et al.,* 2005). SraP and its homolog, GspB, from *S. gordonii* have several common features. GspB has an ability to directly bind to GPIbα on platelets (Bensing *et al.*, 2004). SraP and GspB have same molecular mass, 227 kDa (Siboo *et al.,* 2005). They have atypical signal peptides in their N terminus and a cell wall anchoring motif (LPDTG) in C-terminus. This suggests that SrpA could bind to GPIbα (Siboo *et al.,* 2005).

1.11.1.2 *S. aureus* causes inhibition of platelet activity

Alternatively, *S. aureus* can inhibit platelet activation. For example, *S. aureus* releases lipoteichoic acid (LTA), a surface molecule that plays a role in the interaction with platelets (Waller *et al.*, 2013). LTA shows an ability to suppress activation of platelets through interaction with platelet activating factor receptor (PafR) that is found on the platelet surface (Waller *et al.*, 2013).

Efb has anti-thrombotic properties that inhibits platelet aggregation. The binding site for fibrinogen is located on the N-terminal region of Efb (Shannon *et al.*, 2004). Inhibition of platelet aggregation is presumed to be beneficial for *S. aureus* to inhibit wound healing and so spread the bacterium inside the host body to cause a systemic infection (Athanasopoulos *et al.*, 2006; Shannon *et al.*, 2005).

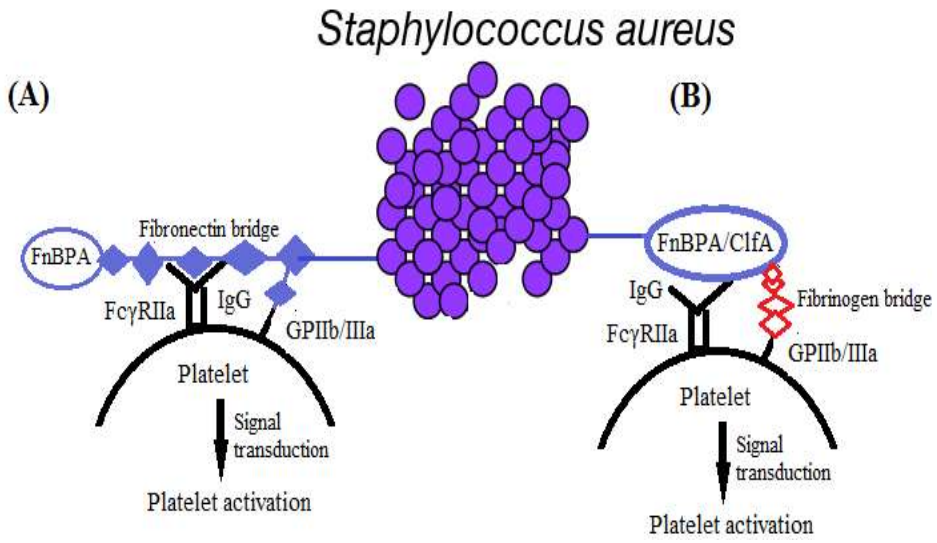

Figure 1.8: Interaction of *S. aureus* with platelets. (A) Fibronectin bridge with FnBPA can cause activation of platelets. **(B)** Platelets also become activated *via* the fibrinogen bridge of ClfA and FnBPA that bind to integrin $\alpha_{IIb}\beta_3$, also known as GPIIb/IIIa. Figure adapted from Fitzgerald *et al.,* (2006).

1.11.2 Interaction of other bacteria with platelets

The human oral cavity is colonised by viridans group streptococci. These bacteria are both successful commensals and opportunistic pathogens of humans through contributing to tooth decay and causing infective endocarditis.

Streptococcus sanguinis, a member of the viridans group streptococci, was reported as one of the major causes of IE in humans (Fowler *et al.*, 2005). The ability of *S. sanguinis* to cause IE was studied using animal models of endocarditis. *S. sanguinis* SrpA showed an ability to adhere to and activate platelets through binding directly to glycoprotein (GP) Ibα, which is an extracellular part of the GPIb-V-IX receptor on platelets (Herzberg *et al.*, 1983; Herzberg *et al.*, 1992). Studies identified platelet-aggregation-associated protein (PAAP), a rhamnose-rich glycoprotein that was isolated from *S. sanguinis*, as mediating platelet adhesion and aggregation (Erickson *et al.*, 1987, 1993). PAAP interacts with the platelet FcγRIIa receptor, leading to platelet aggregation (Sullam *et al.*, 1988).

Streptococcus gordonii is another member of the viridans group streptococci that causes infective endocarditis. Pathogen-platelet interactions occur through binding of its SspA/SspB, GspB and Hsa surface proteins to unknown platelet receptor (Kerrigan *et al.*, 2007) Platelet adherence protein A (PadA) is another protein of *S. gordonii* that is involved in an interaction with platelets. PadA can cause platelet activation by binding to integrin $\alpha_{IIb}\beta_3$ (Haworth *et al.*, 2017).

Streptococcus mitis is commonly found in the throat and mouth of humans. This bacterium can cause IE (Lamas *et al.*, 2003). Proteins PblA and B of *S. mitis* cause platelet aggregation and these surface proteins are encoded on a lysogenic bacteriophage (Bensing *et al.*, 2001). Lysogenic bacteriophage produces lysin and has a role in IE through interaction with fibrinogen that binds to integrin $\alpha_{IIb}\beta_3$ (Seo *et al.*, 2010).

β-hemolytic streptococci have the ability to activate platelets. For example, *Streptococcus agalactiae* is the most common cause of pneumonia and sepsis in neonates (Edwards *et al*., 2011). This bacterium can also cause IE in immunocompromised adult patients (Edwards *et al*., 2010). This bacterium expresses a fibrinogen-binding protein (FbsA) that causes activation of platelets in a similar way to *S. aureus* ClfA (Pietrocola *et al.*, 2005). *Streptococus pyogenes* causes rapid platelet activation (Sjobring *et al.*, 2002). However, the mechanism of this activation is still unknown (Sjobring *et al.*, 2002).

Several Gram-negative bacteria are involved in interactions with platelets. *Porphyromonas gingivalis* is the most common oral bacterium responsible for causing periodontal disease (Griffen *et al.*, 1998) and bacteraemia (Brodala *et al.*, 2005). *P. gingivalis* causes rapid formation of atheroma (Maekawa *et al*., 2011). *P. gingivalis* causes platelet aggregation by its Hgp44 adhesin on the bacterial cell surface. Deletion of genes encoding for Hgp44 leads to *P. gingivalis* losing the ability to aggregate platelets (Naito *et al.*, 2006). *Borrelia burgdorferi* expresses p66 protein that can aggregate platelets (Defoe *et al*., 2001).

Finally, infection with *Bacillus anthracis* or *Bordetella pertussis* leads to hemorrhage. *B. anthracis* expresses oedema and lethal toxins, which inhibit platelet aggregation. The

oedema toxin inhibits aggregation of platelets by activation of PKA (Alam *et al.*, 2006). The lethal toxin suppresses p38 mitogen-activated protein kinase that leads to failure to activate platelets (Kau *et al.*, 2005). *B. pertussis* expresses adenylate cyclase toxin (ACT) which causes inhibition of platelet aggregation. ACT has the same mode of *B. anthracis* oedema toxin (Iwaki *et al.*, 1999).

1.12 Aims of the thesis

CA-MRSA secretes large amounts of PSMs (Li *et al.*, 2010; Wang *et al.*, 2007), and its pathogenicity is strongly related to their secretion. These toxins play a central role in the pathogenesis of *S. aureus* through interaction with circulating blood cells (Cheung *et al.*, 2012; Kretschmer *et al.*, 2010; Schreiner *et al.*, 2013). It is known that platelets are a major part of the circulating blood cell repertoire (Nieswandt *et al.*, 2011), thus this thesis aimed to establish a link between PSMs and their effects on platelets, which had never hitherto been established.

The scientific hypothesis of this thesis was that *S. aureus* PSMs play a role in the interaction of this pathogen with platelets and influences platelet functions.

This thesis was addressing the following questions:

(i) What effect does *S. aureus* PSMs have on human platelet activity?

(ii) What effect does *S. aureus* PSMα3 have on platelet functions, including platelet aggregation, activation of integrin $\alpha_{IIb}\beta_3$, granules release and spreading?

(iii) Does *S. aureus* PSMα3 work dependent or independent on FPR2 in platelets?

(iv) Which signalling pathway in platelets is modulated by *S. aureus* PSMα3?

CHAPTER 2

Materials and methods

Chapter 2

2. Materials and Methods

2.1 Ethics statement

Collection of blood was conducted with the approval from the Research Ethics Committee at the University of Reading. The blood was collected from healthy human volunteers. Informed written consent for collecting blood was provided by volunteers. Blood preparation and platelet isolation were conducted at the Institute for Cardiovascular and Metabolic Research at the University of Reading.

2.2 Solutions

2.2.1 Solutions used in platelet preparation

Table 2.1: Tyrode's solution

Tyrode's solution	
NaCl	134 mM
KCl	3 mM
$Na_2HPO_4.12H_2O$	341 μM
$NaHCO_3$	12 mM
HEPES	20 mM
$MgCl_2.6H2O$	467 μM
pH	7.2

Table 2.2: Citrate

Citrate	
Trisodium citrate $2H_2O$	15.5 mM

Table 2.3: Acid Citrate Dextrose

	Acid Citrate Dextrose
Trisodium citrate $2H_2O$	85 mM
Glucose	111 mM
Citric acid (anhydrate)	78 mM

2.2.2 Solutions used in flow cytometry assays

Table 2.4: HEPES Buffer

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

2.2.3 Solutions used in platelet pulldown assay

Table 2.5: Phosphate Buffered Saline (PBS)

	Phosphate Buffered Saline (PBS) (pH 7.4)
NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	2 mM

Table 2.6: NP40 Lysis Buffer

	NP40 Lysis Buffer
NP40	1%
NaCl	150 mM
Tris-HCl (pH-8)	50 mM
Aprotinin	1.5 mM
Leupeptin	23 μM
Phenylmethylsulfonyl Fluoride	1 mM
Pepstatin A	1.5 μM

Table 2.7: Phosphate Buffered Saline with Tween

Phosphate Buffered Saline with Tween (total volume 1000.5 mL)	
NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Ultrapure water	1000 mL
Tween 20	0.5 mL
pH	7.4

2.2.4 SDS PAGE Solutions

Table 2.8: SDS Sample Buffer

SDS Sample Buffer (10 mL)	
1M Tris/HCl (pH 6.8)	2.5 mL
Glycerol	4 mL
20% SDS	1 g
2-mercaptoethanol	2 mL
0.1% Bromophenol blue	0.8 mL
Ultrapure water	Added to make up final volume

Table 2.9: SDS PAGE Running Buffer

SDS PAGE Running Buffer	
Tris base	124mM
Glycine	960mM
20% SDS	17mM

2.2.5 Western blotting solutions

Table 2.10: Tris Buffered Saline with Tween

Tris Buffered Saline with Tween (1000.5 mL)	
Tris	9.68 g
NaCl	32 g
KCl	0.2 g
Tween 20	0.5 mL
Ultrapure water	1000 mL
pH	7.4

Table 2.11: Western immuno-blot transfer buffer

Western immuno-blot transfer buffer (1000 mL)	
Tris	5.82 g
SDS	0.375 g
Glycine	2.93 g
Methanol	200 mL
Ultrapure water	Added to make up final volume
pH	9.2

Table 2.12: Membrane stripping buffer

Membrane stripping buffer (total volume 90.8 mL)	
SDS	2 g
0.5M Tris/HCl (pH 6.8)	12.5 mL
β-mercaptoethanol	0.8 mL
Ultrapure water	77.5 mL

Table 2.13: Preparation of 12% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) gel

Following recipe was used to prepare 1 mini SDS-PAGE gel.

Component	Separation gel (12%) (w/v)
Double-distilled water (ddH_2O)	3.351 mL
30% Acrylamide	3.999 mL
1.5 M Tris pH 8.8	2.5 mL
10% Sodium dodecyl sulfate (SDS)	100 µL
10% Ammonium persulfate (APS) (MW:228.20)	50.0 µL
Tetramethylethylenediamine (TEMED) (20g)	5.0 µL

Component	Stacking gel (4%) (w/v)
ddH_2O	2.28 mL
30% Acrylamide	0.48 mL
0.5M Tris pH 6.8	0.9 mL
10% SDS	36.0 µL
10% APS	18.0 µL
TEMED	3.6 µL

2.3 Antibodies

2.3.1 Flow cytometry

Table 2.14: Fluorescent antibodies, P: polyclonal, M: monoclonal antibodies.

Assay	Antibody	Clone	Host	Isotype	Working concentration	Company
Fibrinogen binding	FITC Fibrinogen (P)	N/A	Rabbit	IgG	1:200	Dako
P-selectin exposure	CD62P-PE (M)	AK-4	Mouse	IgG1κ	1:200	BD Bioscience

2.3.2 Immunofluorescence Microscopy

Table 2.15: Antibodies used in immunofluorescence microscopy

Assay	Antibody	Clone	Host	Isotype	Working concentration	Company
Localisation of FPR2 in human platelets	anti-FPR2 AlexaFluor 647-labeled (P)	N/A	Rabbit	IgG	1:100	Abnova
Localisation of CD41 (GPIb) in human platelets	anti-GPIb AlexaFluor-488-labeled (M)	MwReg30	Mouse	IgG1κ	1:200	Emfret Analytics

2.3.3 Western blotting antibodies

Table 2.16: Western blotting antibodies

Western blot	Antibody	Host	Working concentration	Isotype	Company
PLC substrate phosphorylation	Phospho-PLC substrate (P)	Rabbit	1:1000	IgG	Cell Signaling Technology
VASP Ser 157	p-VASP antibody – Ser157 (P)	Rabbit	1:1000	IgG	Cell Signaling Technology
VASP Ser 239	p-VASP antibody – Ser239 (P)	Rabbit	1:1000	IgG	Cell Signaling Technology
14-3-3- zeta	14-3-3 ζ Antibody (P)	Rabbit	1:1000	IgG	Santa Cruz Biotech
FPR2 detection	Anti-FPR2 (P)	Rabbit	1:1000	IgG	Abnova
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	Secondary antibody	Rabbit	1:10000	IgG	Novex

2.4 Chemicals and consumables

Table 2.17: Chemicals and consumables used in this thesis

Chemicals and consumables	Catalogue number	Source
24 well culture plates	142475	Thermo Fisher Scientific
6x6 well culture plates	140675	Thermo Fisher Scientific
Acrylamide	A3553	Sigma
Alexa Fluor™ 647 Phalloidin	A22287	Thermo Fisher Scientific
Ammonium persulphate (APS)	A3678	Sigma
Bovine serum albumin (BSA)	BP9702-100	Fisher Scientific
Bovine thrombin	T7513	Sigma
Bromophenol Blue	B0126	Sigma Aldrich
cAMP Enzyme Immunoassay Kit (CA200)	CA200	Sigma-Aldrich
CRP-XL	N/A	Professor R. Farndale (University of Cambridge, UK)
Cyanogen bromide-activated-Sepharose® 4 Fast Flow (CNBr-Sepharose)	C9142	Sigma
DiOC6(3) (3,3'-Dihexyloxacarbocyanine Iodide)	D273	Thermo Fisher Scientific
Donkey serum	D9663	Sigma
Fua-2 AM	F1221	Life Technologies
Glycerol	G5516	Sigma

Glycine	G8898	Sigma
H 89 dihydrochloride	2910	Bio-Techne Ltd
Mini-PROTEAN Tetra Cell Electrophoresis System	1658005	Bio-Rad
MMK-1	3537	Bio-Techne Ltd
Mount media	P36970	Life Technologies
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	T9281	Sigma
PAPA NONOate	sc-202272	Santa Cruz Biotechnologies
Paraformaldehyde (PFA)	P6148	Sigma Aldrich
Pierce™ ECL Western Blotting Substrate	32106	Fisher Scientific
Polyvinylidene difluoride (PVDF) membrane	1704156	Bio-Rad
Prostaglandin I_2	P6188	Sigma
Round coverslips, Poly-L-Lysine coated 12 mm	734-1005	VWR
Rp-8-Br-cAMPS	sc-3539	Santa Cruz Biotechnologie
S. aureus PSMα3	401-004	IBT Bioservices
SilverQuest™ Silver Staining Kit	LC6070	Thermo Fisher Scientific
Sodium Chloride	S7653	Sigma Aldrich

Sodium Dodecyl Sulphate	L3771	Sigma
Sodium Fluoride	450022	Sigma Aldrich
Tris-Base	T4661	Sigma
Tris-HCl	10812846001	Roche
Triton X-100	T8787	Sigma
Tween-20	P9416	Sigma
Vena8™Biochip	V8CF-400-100-02P5	Cellex Ltd
VIP	1911	Bio-Techne Ltd
VIP (6-28)	1905	Bio-Techne Ltd
WRW4	2262	Bio-Techne Ltd

2.5 Washed human platelets preparation

45 mL of blood was collected in a syringe containing 5 mL of 3.8% (w/v) sodium citrate and 7.5 mL of acid-citrate-dextrose (ACD) (85 mM sodium citrate, 71 mM citric acid and 110 mM glucose) (Table 2.3). Blood was transferred into LP4 tubes (75x12 mm, catalogue number: TEP070040, SciChem) and centrifuged at 102 g for 20 minutes at 20ºC. 10 µL PGI_2 (125 µg/mL of PGI_2 solubilized in ethanol) was added to each test tube and centrifuged at 1,413 g for 10 minutes at 20ºC.

The isolated platelets were added to 25 mL of modified 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES)-buffered Tyrode's solution with 3 mL of ACD and 10 µL of PGI_2 (125 µg/mL). Platelets were counted by a coulter cell counter (Beckman Coulter, USA). Platelets were centrifuged at 1,413 g for 10 minutes at 20ºC. The platelet pellet was re-suspended in 1 mL of HEPES-buffered Tyrode's solution. Washed platelets were incubated at 30ºC for 30 minutes prior to their use.

2.6 Platelet rich plasma (PRP) preparation

45 mL of blood was collected in a syringe containing 5 mL of 3.8% (w/v) sodium citrate and 7.5 mL of ACD and centrifuged at 102 g for 20 minutes at 20ºC. Platelets were incubated at 30ºC for 30 minutes before test for recovering their response.

2.7 Platelet aggregometry

450 µL a suspension of platelets (4×10^8 cells/mL) was added into a glass cuvette which was incubated in a Chronolog 490-2d aggregometer at 37ºC for 5 minutes with stirring at 1,200 rpm. Tyrode's buffer (Table 2.1) (25 µL), 25 µL of *S. aureus* PSMα3 (0.8, 3.8, 7.7, 15.38 and 30.7 µM), 25 µl of thrombin (0.05 U/mL) or collagen-related-

peptide-cross-linked (CRP-XL, GCO-(GPO)10-GCOG), GPVI selective agonist, (0.5 µg/mL) were added and recorded by a Chronolog 490-2d aggregometer for 5 minutes.

2.8 *In vitro* thrombus formation under flow

2.8.1 Cellix slide preparation

The cellix slide was prepared by injection of collagen at 400 µg/mL to the loading end side of the chip. It was then incubated at 25ºC for 60 minutes. The opposite side of the cellix capillary was loaded with 20 µL of 1% BSA and incubated at 25ºC for 60 minutes. After incubation, 100 µL of HEPES-buffered Tyrode's solution was added to the loading side of the slide.

2.8.2 Thrombus under flow assay

Incubation of citrated whole human blood with *S. aureus* PSMα3 and appreciated control was carried out for 20 minutes. Then 3,3-dihexyloxacarbocyanine iodide (DIOC6), a lipophilic dye (100 µg/mL), was added to blood treated with appreciated control and *S. aureus* PSMα3. After that, a collagen-coated (400 µg/mL) Vena8™Biochip (Cellex, Dublin) was used for perfusing the treated blood at 20 dynes/cm^{-2} shear rate. Z-stack images of developing thrombi were captured every 30 seconds over 10 minutes by a Nikon Eclipse (TE 2000-U) microscope. The fluorescence intensity of thrombi was analysed by Slidebook ™5 software (Intelligent Imaging Innovations, Denver, USA).

2.9 Flow cytometry

BD Accuri C6 Flow cytometry with an installed software (Becton Dickinson, USA) was used for detecting fibrinogen binding and exposure of P-selectin. FL1-A was used for the fibrinogen binding and FL3-A was used for the exposure of P-selectin.

Polyclonal rabbit anti-human fibrinogen/FITC and PE-CY^{TM}5-labelled mouse anti-human CD62-P antibodies (Table 2.14) were used for detecting fibrinogen binding and P-selectin exposure in response to different concentrations of the *S. aureus* PSMα3.

Each assay contained 50 µL of PRP, 5 µL of *S. aureus* PSMα3 (3.8, 7.7, 15.38, 30.7 µM) and 1 µL of PE-CY^{TM}5-labelled mouse anti-human CD62-P or polyclonal rabbit anti-human fibrinogen/FITC with HEPES-buffered Tyrode's buffer. This was incubated at room temperature for 5 minutes in the dark then reincubated for 20 minutes after adding 0.5 µg/mL of CRP-XL and 0.05 U/mL of thrombin. 25 µg/mL of Gly-Pro-Arg-Pro (GPRP) was added to prevent fibrin polymerisation.

Paraformaldehyde (0.2%) was added to stop the reaction. A BD Accuri C6 Flow cytometer was used to measure and analyse median fluorescence intensity of samples which consisted of 10000 gated events.

2.10 Calcium mobilisation

PRP was incubated with 2 µM/mL of Fura-2 AM dye (Life Technologies, Carlsbad, CA) for 60 minutes at 30ºC. Loaded PRP was centrifuged at 350 g for 20 minutes and re-suspended in HEPES-buffered Tyrode's solution at a density of $4x10^8$ cells/mL. After that, loaded platelets were incubated with *S. aureus* PSMα3 for 5 minutes prior to activation by platelet agonists and recorded for 3 minutes by spectrofluorimetry. A

microplate fluorometer (Thermolab Systems) was used to measure the release of Ca^{2+} at a wavelength of 510 nm for emission and 340 nm and 380 nm for excitation.

2.11 Determination of intracellular cAMP inside the platelet

An ELISA was performed on washed human platelets either treated with or without *S. aureus* PSMα3, using a cAMP Enzyme Immunoassay Kit from (Sigma) according to manufacturer's instructions.

2.12 Clot retraction

Human PRP (200 µL) was mixed with 5 µL of red blood cells, and 15.38 µM or 30.7 µM of *S. aureus* PSMα3. Then 1 mL of HEPES-buffered Tyrode's solution was added to this mixture and incubated for 5 minutes at room temperature. Thrombin, 50 µL to a final concertation of 1 U/mL, was added to this mixture to start clot formation. A glass capillary was placed in the middle of the glass test tube.

Clot formation was monitored and recorded by photography at 0, 30 and 90 minutes. Finally, clots from these glass test tubes were collected and their weight was measured on an electronic balance.

2.13 Immunofluorescence microscopy

Human PRP was isolated from whole blood treated with 3.2% (w/v) sodium citrate. Isolated PRP were fixed by 4% (v/v) paraformaldehyde and incubated for 15 minutes. Then PRP were centrifuged at 950 g for 11 minutes, and 1mL of HEPES-buffered Tyrode's solution was added to resuspend the pellet before centrifugation at 950 g for 10

minutes. The pellet was resuspended in 500 µL of HEPES-buffered Tyrode's solution with 1% protease free BSA. About 120 µL of platelets were added to coverslips (Table 2.17) that were already placed in 6x6 well culture plates (Table 2.17) and incubated for 90 minutes at 37ºC. 6x6 well culture plates were filled with wet tissue in spaces between the wells to generate humidity. Phosphate buffered saline (PBS), was used to wash non-adhered platelets. Each washing step (PBS) was repeated 3 times for 2 minutes. After that 0.2% (v/v) Triton X-100, 1% (w/v) BSA and 2% (v/v) donkey serum (Table 2.17) were used for blocking the reaction on the coverslips and incubated at room temperature for 1 hour. After that, platelets were incubated with primary antibody that was added to permeabilisation solution which contained 0.2% (v/v) Triton X-100, 1% (w/v) BSA and 2% (v/v) donkey serum. Then three times of washing with 1X PBS for removing unbound antibody.

Secondary antibody was incubated with platelets that were fixed on coverslips at room temperature for 1 hour. Removal of unbound antibody through washing 3 times with PBS was performed. After that 4% paraformaldehyde was used to fix the platelets for 5 minutes. The coverslips were washed with 1X PBS. Finally, a small drop of mount media (Table 2.17) was placed on each coverslip. For visualisation of platelets under 100X oil immersion lens, Nikon Al-R confocal microscope with Alexa Fluor filters at 488 nm and 647 nm was used.

2.14 Platelet spreading

200 µL of fibrinogen (100 µg/mL) was used for coating a 24 well culture plate (128 mm X 86 mm, 1.9 cm^2) with coverslips inside each well. The coated coverslips were incubated for 1 hour at 37ºC. After that 200 µL of PBS was used to wash the coverslips

twice. Then 1% (w/v) BSA–PBS solution was used for blocking and incubated at room temperature for 1 hour. Then coverslips were washed twice with 200 µL of PBS. After that, 200 µL of washed platelets ($2x10^5$ cells/mL) were incubated with 30.7 µM of *S. aureus* PSMα3 for 15 minutes at 37ºC. Then 200 µL of platelets treated with 30.7 µM of *S. aureus* PSMα3 were transferred into wells containing the fibrinogen coated coverslips. After that incubation of platelets with 30.7 µM of *S. aureus* PSMα3 was carried out based on time course, 30, 45 and 60 minutes at 37 ºC. Washing of platelets was carried out with HEPES-buffered Tyrode's solution and platelets were then fixed with 4% (v/v) of paraformaldehyde and incubated for 10 minutes at 37ºC. Then platelets were washed twice with 200 µL of PBS and permeabilisation solution was added to platelets for 10 minutes at 37ºC.

Platelets were washed with 200 µL of 1X PBS twice. Platelets were stained with 200 µL of Alexa Fluor™ 647 Phalloidin (Table 2.17) with dilutions of 5:200 in 1% (w/v) BSA–PBS, then incubated in the dark at room temperature for 20 minutes. Then 2 washing cycles with 200 µL of PBS were carried out on these platelets. Finally, coverslips were taken out from the wells, mounted with a small drop of mount media and then placed onto glass slides. A Nikon Al-R confocal microscope was used to analyse theses slides.

2.15 Western immuno-blotting

2.15.1 Preparation of sample

Platelet lysates ($4x10^8$ cells/mL) were incubated with 15.38 µM or 30.7 µM of *S. aureus* PSMα3 with or without stimulation by CRP-XL or thrombin for 90 seconds in stirring conditions at 37°C for 5 minutes. Human platelets were lysed in 6X Laemmli sample buffer and boiled for 10 minutes at 95°C.

2.15.2 SDS-PAGE

Extracted proteins from platelet lysates, as described in section 2.15.1, were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). These proteins were loaded onto a 12% (w/v) acrylamide gel that was run in a Mini Protean Electrophoresis System (BioRad) containing running buffer that was described in Table 2.9 at a constant voltage of 200 mA for 90 minutes at 25ºC.

2.15.3 Western blotting

After that, separated proteins were transferred to PVDF membrane (Table 2.17) and 2% (w/v) BSA in 1X Tris-buffered saline (TBS) and Tween 20 (TBS-T) solution was added to the membrane for blocking and incubated for 60 minutes at 25ºC. Then primary antibody was added to the membrane and incubated overnight at 4°C. After that, membrane was washed 3 times in 1X TBS-T solution for 15 minutes with gentle shaking and then secondary antibody was added and incubated for 60 minutes. Then, membrane was washed 3 times for 15 minutes by 1X TBS-T solution. Membrane was then incubated with enhanced chemiluminescence (ECL) substrate and visualised by G: BOX Chemi (Syngene, Cambridge, UK).

2.16 Silver staining

SilverQuest™ Silver Staining Kit (Thermo Fisher Scientific) was used according to manufacturer's instructions. Following SDS-PAGE, the gel was removed from the cassette and placed into a tray for staining. The gel was incubated with fixative solution for 20 minutes at room temperature. Then washing of the gel with 30% ethanol for 10 minutes. After that, sensitising solution was added to the washed gel and incubated for 10 minutes. Following that, the gel was washed for 10 minutes by 30% ethanol and the

process was followed by washing through using ultrapure water for 10 minutes. Staining solution was added to the gel and incubated for 15 minutes. Gels were then washed for 60 seconds by ultrapure water. Finally, the washed gel was incubated for 8 minutes with developing solution. After that, bands appeared and the reaction was stopped upon addition of stopper solution.

2.17 Coupling *S. aureus* PSMα3 to CNBr-Sepharose Fast Flow

S. aureus PSMα3 was coupled to CNBr-Sepharose Fast Flow as per the manufacturer's instructions. The coupling density of *S. aureus* PSMα3 was 82.14 µg/mL in the resin. The process of coupling was done at 4ºC overnight. Following that, excess reactive groups were quenched by incubation with ethanolamine 1 M (pH 8.0) at room temperature for 2 hours. Then *S. aureus* PSMα3 coupled to CNBr-Sepharose was stored in PBS at 4ºC.

2.18 Co-precipitation assay

Washed platelets at ($8X10^8$ cells/mL) were lysed by adding NP40 lysis buffer (Table 2.6) and immediately placing them on ice. After centrifugation of lysed platelet suspensions at 10000 g for 2 minutes, they were mixed with 50 µL of *S. aureus* PSMα3 coupled to CNBr-Sepharose and incubated at room temperature for 30 minutes under vigorous agitation. After this, pellets were collected by centrifugation of the resins at 3500 g for 2 minutes. The pellets were washed three times in 1 mL of PBST and resuspended in 100 µL of 6X Laemmli sample buffer and heated for 5 minutes at 95°C. Finally, the samples were run on a 12% SDS-PAGE, followed by sliver staining (Table 2.17) for visualisation.

2.19 Mass spectrometry analysis

2.19.1 Preparation of samples for mass spectrometry analysis

Desired bands of protein samples were cut out from the SDS-PAGE gel stained with silver stain. This process was done very carefully to avoid contamination from keratin. After that, destainer solutions in SilverQuest™ Silver Staining Kit (Thermo Fisher Scientific) were used to remove the silver ions from each band, according to the manufacturer's protocol.

2.19.2 Mass Spectrometry Analysis

An Orbitrap Mass Spectrometer (Mass Spectrometry Facility, University of Birmingham, UK) was used for analysing bands. Identification of proteins in the samples was carried out by electron transfer determination in bottom up LC (Liquid Chromatography) MS/MS.

2.20 Bioinformatics analysis

2.20.1 Secondary structure prediction and potential binding sites of *S. aureus* PSMα3

The amino acid sequence of *S. aureus* PSMα3 (Table 2.18-A) was used to predict the secondary structure and possible binding sites within *S. aureus* PSMα3 using Iterative Threading ASSEmbly Refinement (I-TASSER) from (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). I-TASSER provided the predicted peptide structure of 23 amino acid residues using the first identified structure of this peptide by Local Meta-Threading-Server (LOMETS). Following this, the structure of the *S. aureus* PSMa3 was generated into the 3D model by BioLiP.

Table 2.18: Amino acid sequences of *S. aureus* PSMα3 and VPAC1 receptor

Table 2.18-A

***S. aureus* PSMα3**

Amino acid sequence	fMEFVAKLFKFFKDLLGKFLGNN

Table 2.18-B

VPAC1 receptor

Region	Amino acid sequence of VPAC1 receptor
N-terminus	MRPPSPLPARWLCVLAGALAWALGPAGGQAAR LQEECDYVQMIEVQHKQCLEEAQLENETIGCSK MWDNLTCWPATPRGQVVVLACPLIFKLFSSIQG RNVSRSCTDEGWTHLEPGPYPIACGLDDKAASL DEQQTMFYG
TM-1	SVKTGYTIGYGLSLATLLVATAILSLFRKLHC
TM-2	TRNYIHMHLFISFILRAAAVFIKDLALFDSGESD
TM-3	QCSEGSVGCKAAMVFFQYCVMANFFWLLVEGL YLYTLLAV
TM-4	SFFSERKYFWGYILIGWGVPSTFTMVWTIARIHF EDYG
TM-5	CWDTINSSLWWIIKGPILTSILVNFILFICIIRILLQ K
TM-6	LRPPDIRKSDSSPYSRLARSTLLLIPLFGVHYIMF AFFPDNF
TM-7	KPEVKMVFELVVGSFQGFVVAILYCFLNGEVQA ELRRKWRRW
C-terminus	HLQGVLGWNPKYRHPSGGSNGATCSTQVSMLT RVSPGARRSSSFQAEVSLV

2.20.2 Physiochemical properties prediction of *S. aureus* PSMα3

The physiochemical properties of *S. aureus* PSMα3 were analysed by ProtPram (http://web.expasy.org/protparam). The ProtParam provided physical and chemical parameters of *S. aureus* PSMα3 that include molecular weight, composition of amino acids, estimated half-life, and atomic composition.

2.20.3 Modelling of VPAC1 receptor interaction

The amino acid sequence of VPAC1 receptor (Table 2.18-B) was submitted to IntFOLD3.0 (McGuffin *et al.*, 2015; Roche *et al.*, 2011). The predicted model was selected based on the top scored model with a quality score of ≥ 0.788 over 1.0, probability of incorrectness or P-value of ≤ 2.328E-4 and reliability score.

Megadock software (Ohue *et al.*, 2014) was used for docking *S. aureus* PSMα3 into VPAC1 receptor. The quality of the docking model was finally checked by using ProQdock (Basu *et al.*, 2016). It is a scoring program for protein-protein docking, which uses support vector machine (SVM). SVM combines the structural information, the predicted features and scoring functions for providing a score according to the interaction strength (Basu & Wallner, 2016).

2.21 Preparation of bacteria

Table 2.19: List of bacterial strains used

Strain	Phenotype	Source
S. aureus SH1000	Wild type	(Horsburgh *et al*., 2002)

2.21.1 Media

Brain Heart Infusion (BHI) medium was obtained from Oxoid (Basingstoke, UK) and it was prepared as described by manufacturer's instructions. Agar was added where appropriate. All glassware was heat sterilised at 150°C for 2.25 hours prior to use in microbiological procedures.

2.21.2 Growth conditions

S. aureus was inoculated into BHI medium for 16 hours at 37°C with shaking at 260 rpm. The OD_{600} of the culture was taken to confirm that the cultures were in post exponential phase. Then 1% of the previous culture was inoculated into BHI medium at 37°C with shaking at 260 rpm for 16 hours. The OD_{600} was taken every 20 minutes to produce a growth curve.

2.21.3 Preparation of whole bacteria for aggregation assays

S. aureus was inoculated into 20 mL plastic universals which contained 5 mL of BHI medium. Afterwards the culture was incubated at 37°C with shaking at 260 rpm for 16

hours until the OD_{600} reached 3.0. Finally, bacteria were washed three times in Tyrodes buffer. The OD_{600} was adjusted to 1 in Tyrodes buffer that is equal to a concentration of $6x10^8$ bacteria/mL.

2.22 Statistical analysis

Statistical significance of data that was collected from assays were assessed by one-way analysis of variance (ANOVA) or Student's t-test and performed by Graph Pad Prism 6 software (San Diego, CA, USA). P values less than 0.05 were considered significant.

CHAPTER 3

Effects of *S. aureus* PSMα3 on platelet functions

3. Effects of *S. aureus* PSMα3 on platelet functions

3.1 Introduction

S. aureus PSM toxins are a relatively recent discovery. They play multifunctional roles during infection (Wang *et al.*, 2007), acting as chemoattractants for human neutrophils and also have the capacity to lyse them (Kretschmer *et al.*, 2010). PSMs also have observable effects on several human cells such as erythrocytes, osteoblasts and epithelial cells (Cassat *et al.*, 2013; Cheung *et al.*, 2012; Giese *et al.*, 2011), although the pattern of lysis in neutrophils is not the same as compared to other cells such as erythrocytes and DCs. PSM toxins contribute to the establishment of large, invasive abscesses in skin and soft tissue during CA-MRSA infection (Wang *et al.*, 2007).

S. aureus PSMα3 shows a stronger lytic activity towards neutrophils when compared to other classes of PSM toxins, due to its large hydrophobic moment (Cheung *et al.*, 2014; Laabei *et al.*, 2014). Moreover, this toxin has a significant role in the pathogenesis of highly hyper-virulent strains of CA-MRSA (Wang *et al.*, 2007).

In this chapter, platelet aggregation assays were performed to look at the effect of *S. aureus* PSMα3 on platelets. Thrombin and CRP-XL were used as positive controls for *in vitro* platelet activation and aggregation, as both target different activation pathways. Platelet aggregation assays measure the response of platelets to different agonists which cause *in vitro* activation and aggregation of the platelets (Varga-Szabo *et al.*, 2008).

Various parameters were used in subsequent experiments e.g. different PSMα3 concentrations, numbers of platelets or bacteria, incubation periods, washed platelets vs. PRP, concentration of agonist or antagonist. These experiments were addressing the following questions:

- The effects of PSMα3 were measured on platelet activity at a broad range of concentrations (0.1 μM to 30.7 μM), because the amount of PSMα3 produced in culture supernatant by CA-MRSA ranged from 0.1 μM to 20 μM (Yamaki *et al.*, 2011).

- In stirred aggregometry experiments, washed human platelets ($4x10^8$ cells/mL) or *S. aureus* SH1000 cells ($6x10^8$ CFU) was used in experiments, because these numbers of platelets or bacteria have been optimised in the laboratory (Waller *et al.*, 2013).

- Incubation periods of PSMα3, agonists and inhibitors were between 5 to 10 minutes in following experiments in this thesis, because their maximum effects are generally observed within 5 to 10 minutes (Goodall *et al.*, 2004).

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- Testing of the ability of PSMα3 to modulate platelet activity was initially done using washed platelets to avoid any interaction with the plasma compounds. Then, the ability of PSMα3 was tested in PRP several experiments, because PSMα3 interacts with plasma compounds such as lipoproteins (Surewaard *et al.*,

2012). Moreover, using washed platelets or PRP was dependent on which was being conducted experiment. For example, washed platelets has a low amount of fibrinogen than is found in PRP that does not cause sustained aggregation. Also the choice of agonist used depended on the experiment. For instance, *S. aureus* SH1000 was tested in PRP, because bacteria need plasma proteins to cause platelet aggregation.

- The concentration of agonist or antagonist used in experiments varied to allow an incorporation of the response of platelets from different donors. For example, EC_{80} for thrombin was used in a range between 0.05 and 0.07 U/mL, which provided around 80% of platelet aggregation in response to thrombin, while 80% of platelet aggregation in response to CRP-XL occurred around 0.5 to 0.6 µg/mL. These lower concentrations of CRP-XL and thrombin were found to cause full platelet aggregation.

3.2 Results

3.2.1 *S. aureus* PSMα3 did not cause platelet activation

In order to test the effect of *S. aureus* PSMs on human platelet activity, the highest concentration of PSMα3 (30.7μM) was added to washed human platelets ($4x10^8$ cells/mL) in the absence of agonists, thrombin or CRP-XL (Figure 3.1). Thrombin and CRP-XL were used as positive controls for *in vitro* platelet activation. Platelets treated with Tyrode's buffer were the negative control.

The effect of the toxin on human platelets was directly recorded in an aggregometer for 5 minutes at 37 °C in stirred conditions, described in 2.7. These data showed that *S. aureus* PSMα3 did not cause activation and aggregation of platelets, unlike thrombin or CRP-XL, which were able to induce platelet activation and aggregation (Figure 3.1). Unexpectedly, given the ability of this toxin to lyse human cells (e.g. neutrophils), addition of PSMα3 did not cause platelet activation (Cassat *et al.*, 2013; Cheung *et al.*, 2012; Giese *et al.*, 2011; Kretschmer *et al.,* 2010).

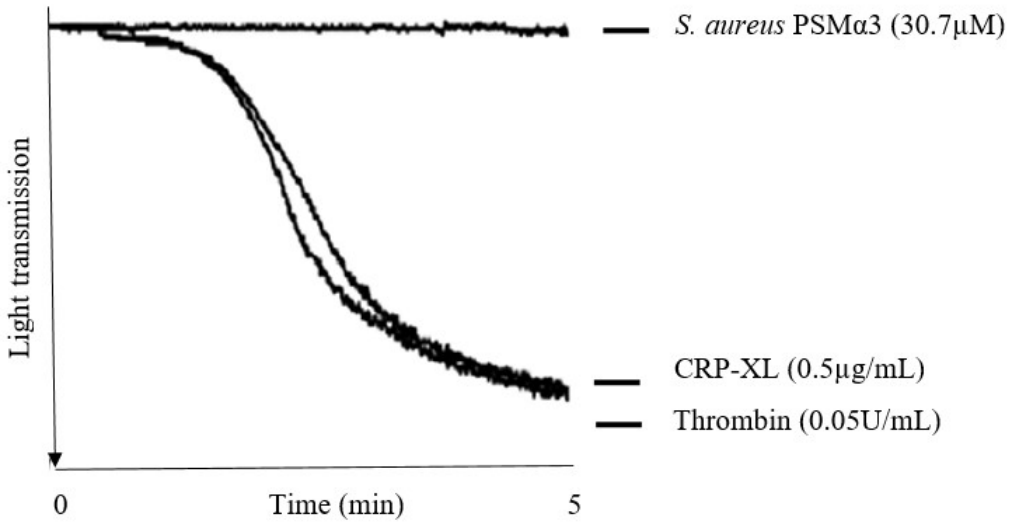


Figure 3.1: Measurement of the effect of *S. aureus* PSMα3 on human platelet aggregation. Representative aggregation traces. *S. aureus* PSMα3 was added to washed human platelets ($4x10^8$ cells/mL) and directly recorded *via* a chronolog 490-2d aggregometer for 5 minutes without adding any platelet agonists. The result showed that *S. aureus* PSMα3 did not cause platelet activation and aggregation cf thrombin and CRP-XL at an OD 405 nm (n=3).

3.2.2 Inhibition of platelet aggregation by *S. aureus* PSMα3

3.2.2.1 Effect of *S. aureus* PSMα3 on different agonists-induced platelet aggregation

Following the experiment in section 3.2.1 which showed that PSMα3 did not activate platelets, further work was done to see the effect of this toxin on platelets in the presence of physiological agonists such as thrombin or CRP-XL. This assay mimics the fundamental role of platelets in human physiology. Washed human platelets or PRP were pre-incubated with *S. aureus* PSMα3 for 5 minutes. Thrombin or CRP-XL was added and recorded by a chronolog 490-2d aggregometer for 5 minutes as described in section 2.7.

Washed human platelets ($4x10^8$ cells/mL) were pre-incubated with different concentrations (0.1. 3.8, 7.7, 15.38 and 30.7 μM) of PSMα3 for 5 minutes before activation with 0.05 U/mL of thrombin (Figure 3.2). Afterwards, washed human platelets were stimulated with thrombin in the presence of increasing concentrations of PSMα3 for 5 minutes. The result showed that PSMα3 has an inhibitory effect on platelet aggregation mediated by thrombin (Figure 3.2A). The percentage aggregation of washed platelets caused by 0.05 U/mL of thrombin was 80% (Figure 3.2B). Aggregation induced by thrombin (0.05 U/mL) was reduced by 70%, 56%, 45%, 30% and 12% at 0.1, 3.8, 7.7, 15.38 and 30.7μM of PSMα3, respectively (Figure 3.2B). The time for platelets to aggregate (known as the lag time) was 2 seconds in response to thrombin, whereas *S. aureus* PSMα3 increased the lag time to 260 seconds in response to 30.7μM of PSMα3 (Figure 3.2C). *S. aureus* PSMα3 was found to cause a concentration-dependent inhibition in platelet aggregation stimulated with thrombin.

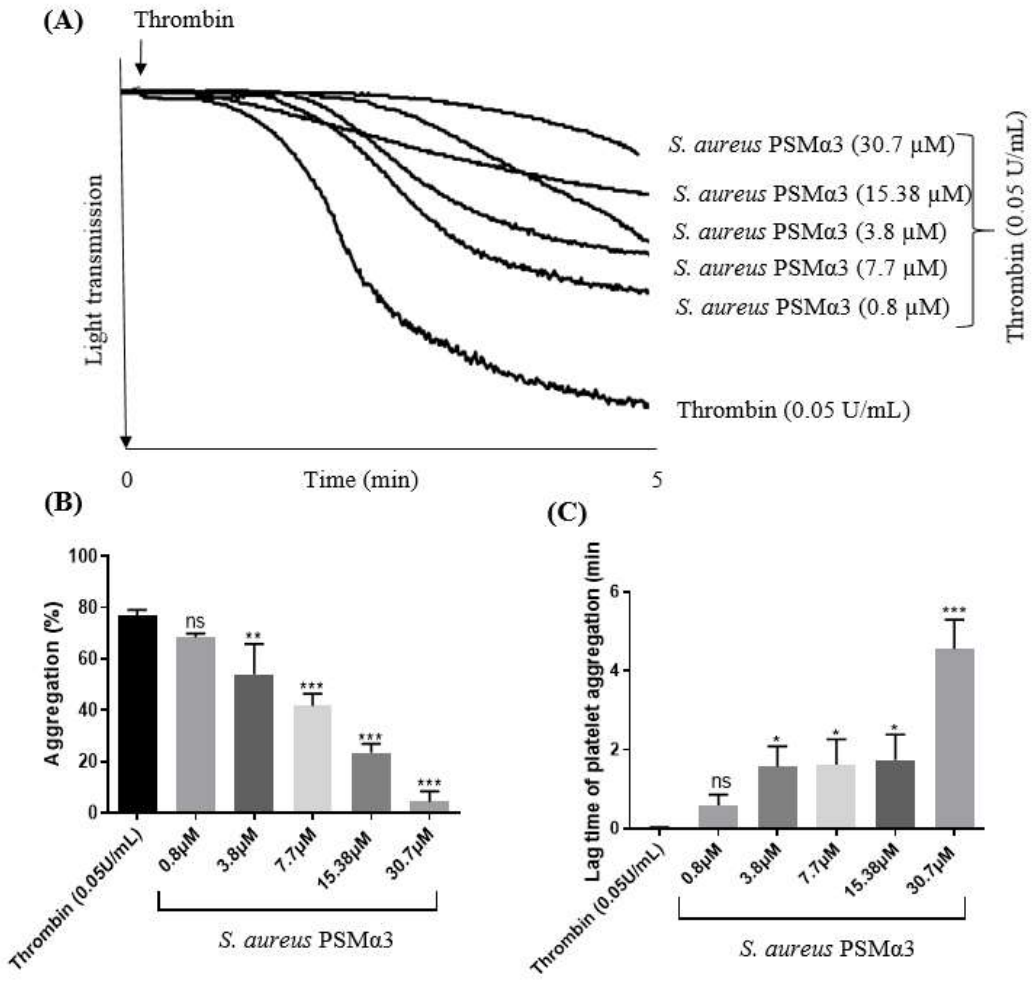


Figure 3.2: *S. aureus* PSMα3 inhibits platelet aggregation mediated by thrombin. **(A)** Representative aggregation traces. Washed human platelets ($4x10^8$ cells/mL) were pre-incubated with different concentrations of *S. aureus* PSMα3 or Tyrode's buffer for 5 minutes before added 0.05 U/mL of thrombin and aggregation recorded by a chronolog 490-2d aggregometer for 5 minutes at OD 405 nm. **(B)** Percentage of platelet aggregation in response to thrombin after 5 minutes. *S. aureus* PSMα3 (30.7 μM) showed the ability to reduce the aggregatory response to thrombin (0.05 U/mL). **(C)** The lag time of platelet aggregation in response to thrombin. A high concentration of *S. aureus* PSMα3 showed a delayed time of platelet aggregation caused by 0.05 U/mL of thrombin. These data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, *P≤0.05, **P≤0.01, ***P≤0.001).

S. aureus PSMα3 inhibited aggregation mediated by thrombin as shown in (Figure 3.2). Therefore, the response to CRP-XL, a GPVI-selective agonist, was tested as it has a different pathway of activation than thrombin (Asselin *et al.*, 1999; Gibbins *et al.*, 1997). The effect of *S. aureus* PSMα3 on platelets stimulated with CRP-XL was measured. The result showed that PSMα3 showed an ability to inhibit platelet activation by CRP-XL (Figure 3.3A). The percentage aggregation was calculated. PSMα3 (30.7µM) reduced the aggregation to 5% cf CRP-XL at 5 minutes (Figure 3.3B). The lag time was increased in platelets treated with *S. aureus* PSMα3 in a dose-dependent manner (Figure 3.3C). For example, the lag time of platelets in response to CRP-XL (0.5µg/mL) without pre-incubated them with PSMα3 was 14 seconds, whereas the time for pre-incubated platelets with PSMα3 to aggregate was 210 seconds (Figure 3.3C). This finding was found to be consistent with inhibition of thrombin-induced platelet aggregation by *S. aureus* PSMα3 (Figure 3.2).

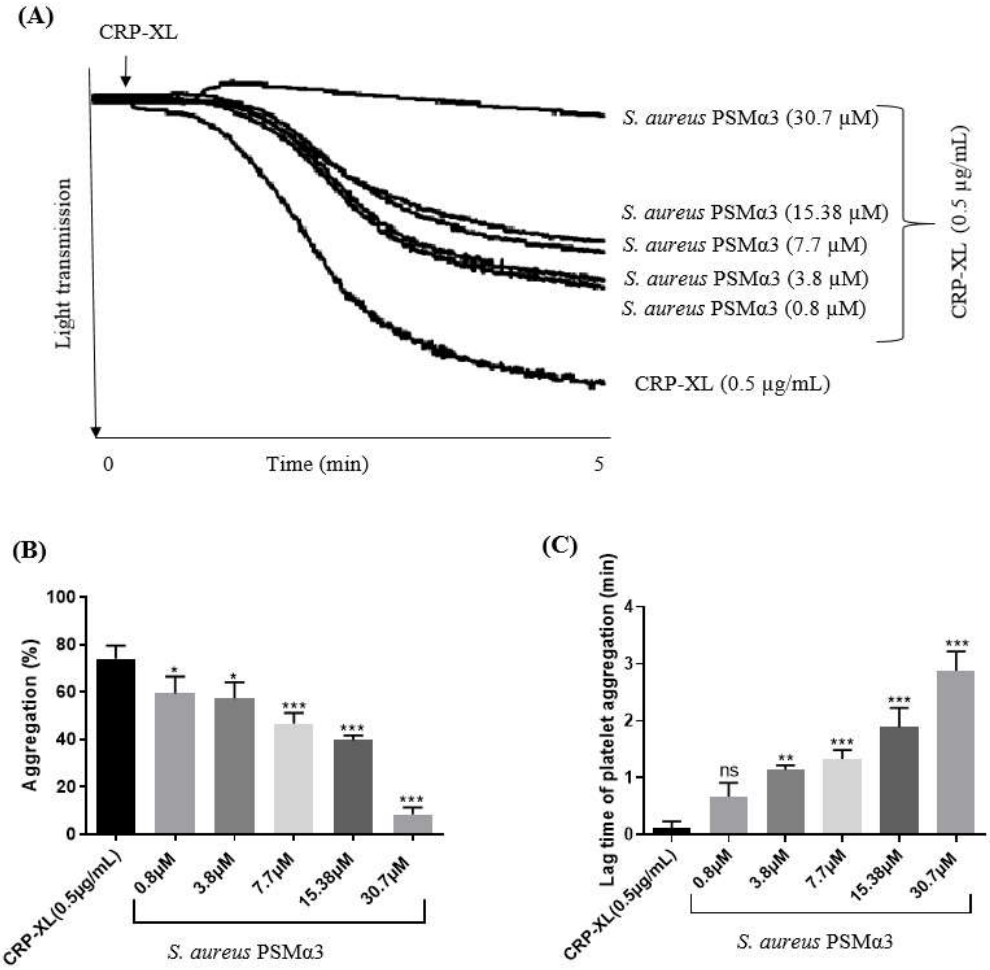
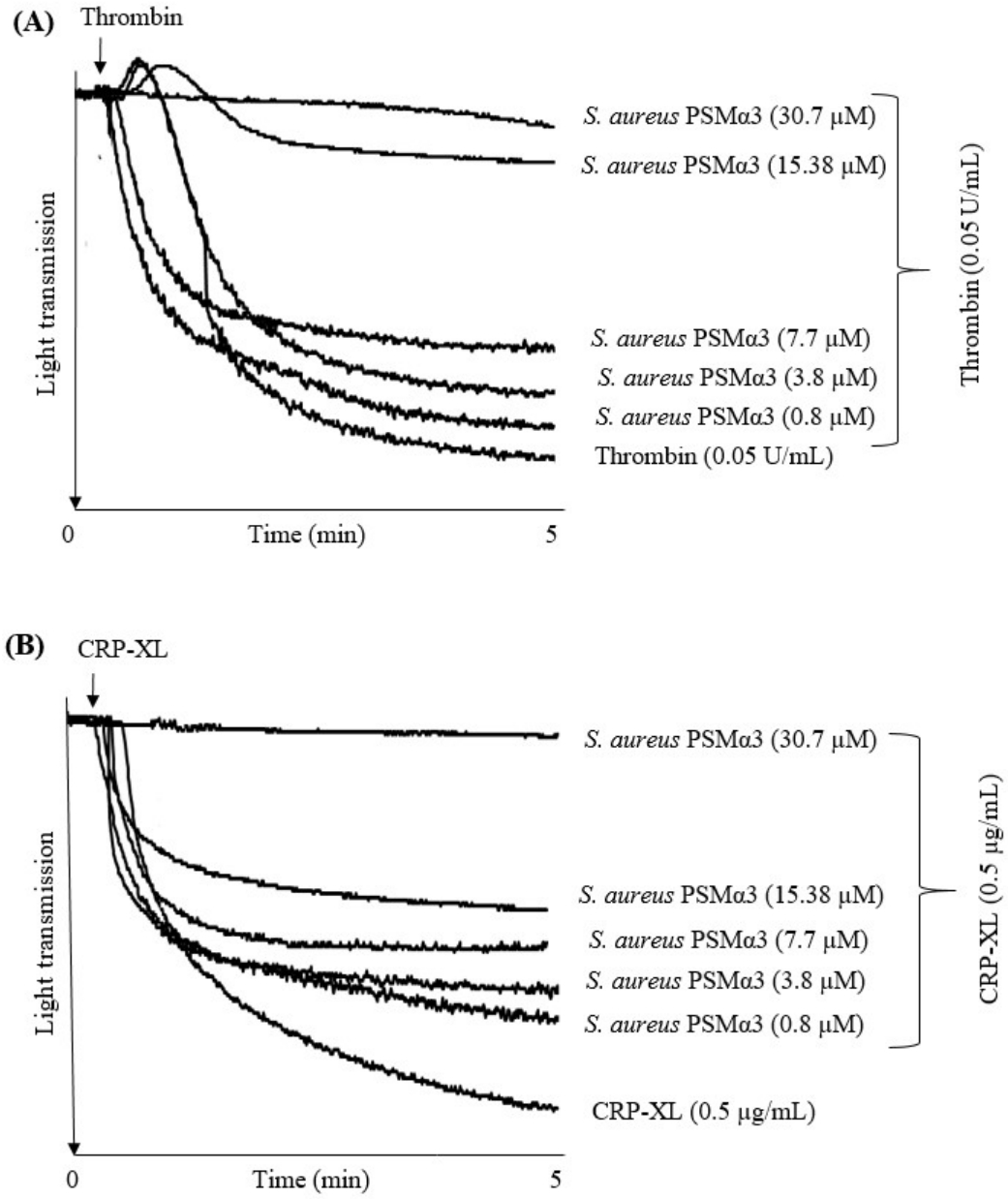


Figure 3.3: *S. aureus* PSMα3 inhibits platelet aggregation mediated by CRP-XL. **(A)** Representative aggregation traces. The reaction of washed platelets treated with different concentrations of *S. aureus* PSMα3 followed by 0.5 µg/mL of CRP-XL and aggregation recorded *via a* chronolog 490-2d aggregometer for 5 minutes. **(B)** Percentage of platelet aggregation. CRP-XL was used to induce platelet aggregation following incubation with different concentrations of *S. aureus* PSMα3. **(C)** Lag time of platelet aggregation mediated by CRP-XL. These data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, *P≤0.05, **P≤0.01, ***P≤0.001).

Because *S. aureus* PSMα3 interacts with plasma proteins such as lipoproteins (Surewaard *et al.*, 2012), the effects of PSMα3 on platelet activity in PRP was studied to compare its impact upon washed platelets. As with washed platelets, thrombin (0.05 U/mL) induced aggregation in PRP was inhibited by PSMα3 in a dose-dependent manner (Figure 3.4A). Aggregation induced by CRP-XL (0.5 µg/mL) was also inhibited by PSMα3 at different concentrations (0.1, 3.8, 7.7, 15.38 and 30.7µM) (Figure 3.4B). The percentage of aggregation in PRP was reduced (Figure 3.4C and 3.4E). Compared with the platelet aggregation studies in washed platelets, 30.7 µM PSMα3 inhibited the aggregation induced by thrombin (0.05 U/mL) at 5 minutes was 8% (Figure 3.2B) whereas in PRP this was increase to 12% (Figure 3.4C). This observation was within the error range. PSMα3 was found to cause a concentration-dependent reduction in CRP-XL (0.5 µg/mL) induced aggregation in PRP at 5 minutes (Figure 3.4B and 3.4E). Moreover, the lag time was increased in PRP treated with *S. aureus* PSMα3, in a dose-dependent manner (Figure 3.4D and 3.4F). This finding was consistent with inhibition of thrombin-induced platelet aggregation by *S. aureus* PSMα3 (Figure 3.2 and 3.3).



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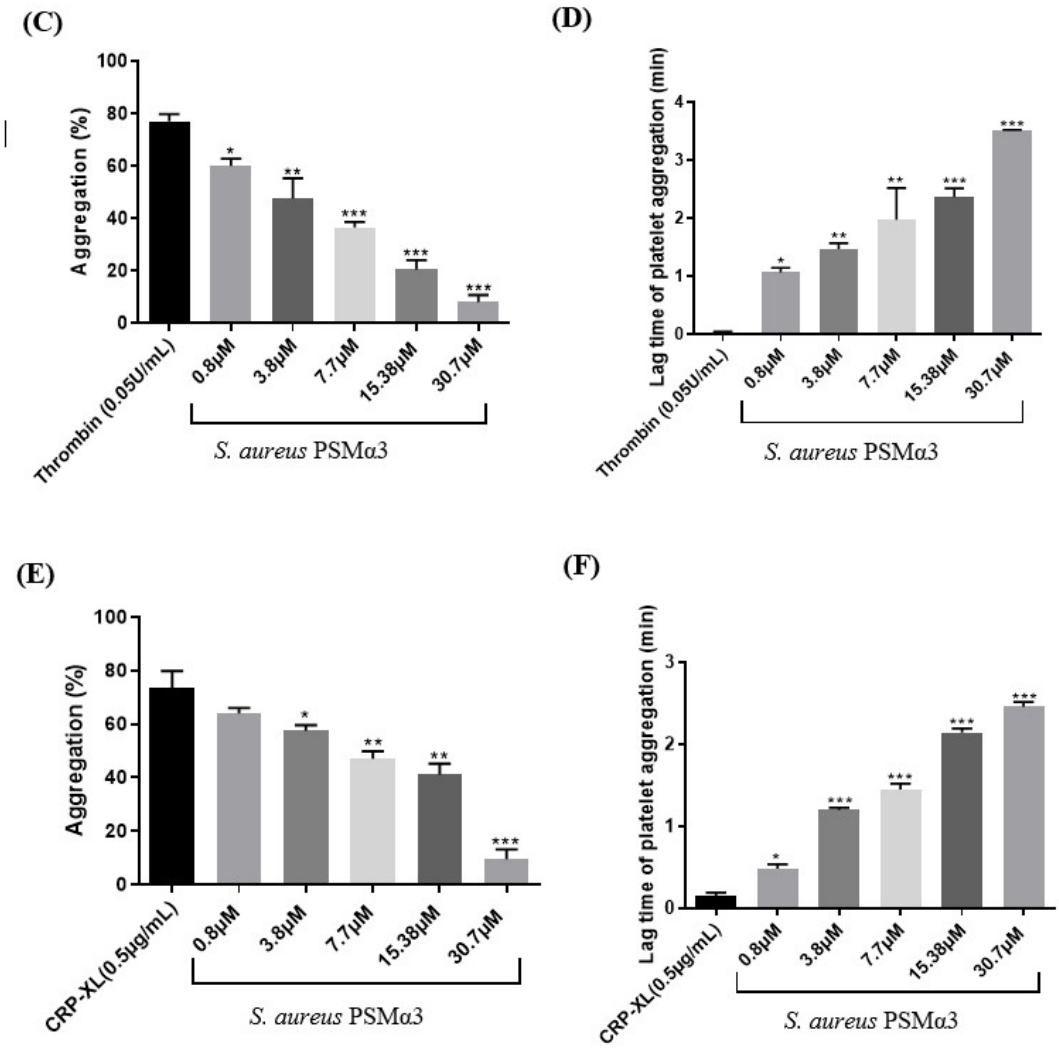


Figure 3.4: *S. aureus* PSMα3 inhibits platelet aggregation in PRP. Representative aggregation traces. PRP was treated with different concentrations of *S. aureus* PSMα3 followed by thrombin **(A)** or CRP-XL **(B)** and aggregation recorded *via a* chronolog 490-2d aggregometer for 5 minutes. **(C, E)** Percentage of platelet aggregation mediated by thrombin or CRP-XL. **(D, F)** Lag time of platelet aggregation mediated by thrombin or CRP-XL. These data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, *P≤0.05, **P≤0.01, ***P≤0.001).

Following from the previous experiments, an ability of PSMα3 to inhibit platelet aggregation mediated by thrombin and CRP-XL was observed, so further work was carried out to see the effect of the PSMα3 on platelets in the presence of whole *S. aureus* bacteria.

Platelet aggregations were done with PRP, using various concentrations of PSMα3 and washed whole *S. aureus* SH1000 cells ($6x10^8$ bacteria/mL). 450 µL of PRP were pre-incubated with various concentrations of PSMα3 for 10 minutes followed by stimulation with 50 µL of whole *S. aureus* SH1000 cells ($6x10^8$ bacteria/mL) of wild type strain. Aggregations were measured for up to 20 minutes. Platelet poor plasma (PPP) was used as a blank.

These data showed that PSMα3 is able to inhibit platelet aggregation mediated by *S. aureus* SH1000 (Figure 3.5A). The percentage aggregation of platelets caused by *S. aureus* SH1000 was 84% (Figure 3.5B) and this was reduced to 10% in platelets treated with 30.7µM of PSMα3 (Figure 3.5B). The lag time of platelets in response to *S. aureus* SH1000 without pre-incubated them with PSMα3 was 13 minutes (Figure 3.5C), whereas the time for platelets pre-incubated with PSMα3 (30.7 µM) to aggregate was, 16 minutes (Figure 3.5C). This finding was consistent with inhibition of platelet aggregation mediated by thrombin and CRP-XL.

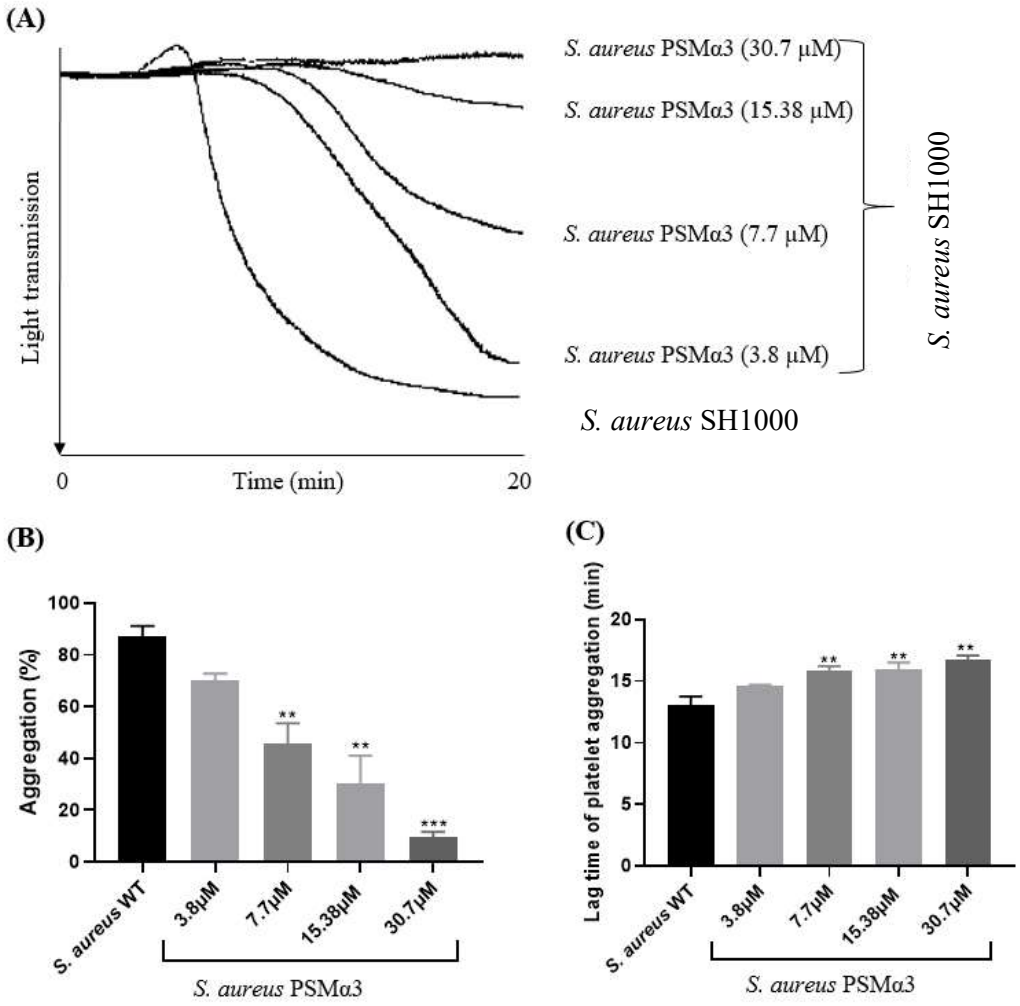


Figure 3.5: *S. aureus* PSMα3 inhibits platelet aggregation mediated by whole *S. aureus* SH1000 bacteria. (A) Representative aggregation traces. Platelets were treated with different concentrations of *S. aureus* PSMα3 followed by stimulation with whole *S. aureus* SH1000 cells ($6x10^8$ bacteria/mL) of wild type strain. Aggregation was recorded *via* chronolog 490-2d aggregometer for 20 minutes. **(B)** Percentage of platelet aggregation. **(C)** Lag time of platelet aggregation. These data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, *P≤0.05, **P≤0.01, ***P≤0.001).

3.2.2.2 Effect of *S. aureus* PSMα3 on platelet aggregation under *in vivo* shear forces

Previous studies showed an inhibitory effect of *S. aureus* PSMα3 on washed platelets, but the assay is not representative of physiological conditions. *In vivo*, platelets experience shear forces. Therefore, a thrombus formation under flow assay was conducted to determine the effect of *S. aureus* PSMα3 on platelet aggregation under *in vivo* shear rates in a condition that mimics the bloodstream.

Platelets in citrated whole human blood were treated with lipophilic dye, 3,3 dihexyloxacarbocyanine iodide (100 µg/mL) (Table 2.17). Then the blood was applied at a shear rate of 20 dynes/cm^{-2} through a collagen-coated (400 µg/mL) Vena8™ Biochip (Cellex, Dublin) (Table 2.17). A Nikon eclipse (TE 2000-U) microscope was used to capture the Z-stack images of thrombi forming every 30 seconds over 10 minutes. Slidebook™5 software (Intelligent Imaging Innovations, Denver, USA) was used to analyse the intensity of thrombus fluorescence.

S. aureus PSMα3 reduced thrombus formation (Figure 3.6 A). It caused a significant decrease in thrombus size (Figure 3.6 B) and approximately ~57% reduction in the peak fluorescence of thrombus formation cf the vehicle treated control (Figure 3.6 C). This result was consistent with previous aggregometry studies that are shown in section 3.2.2.1.

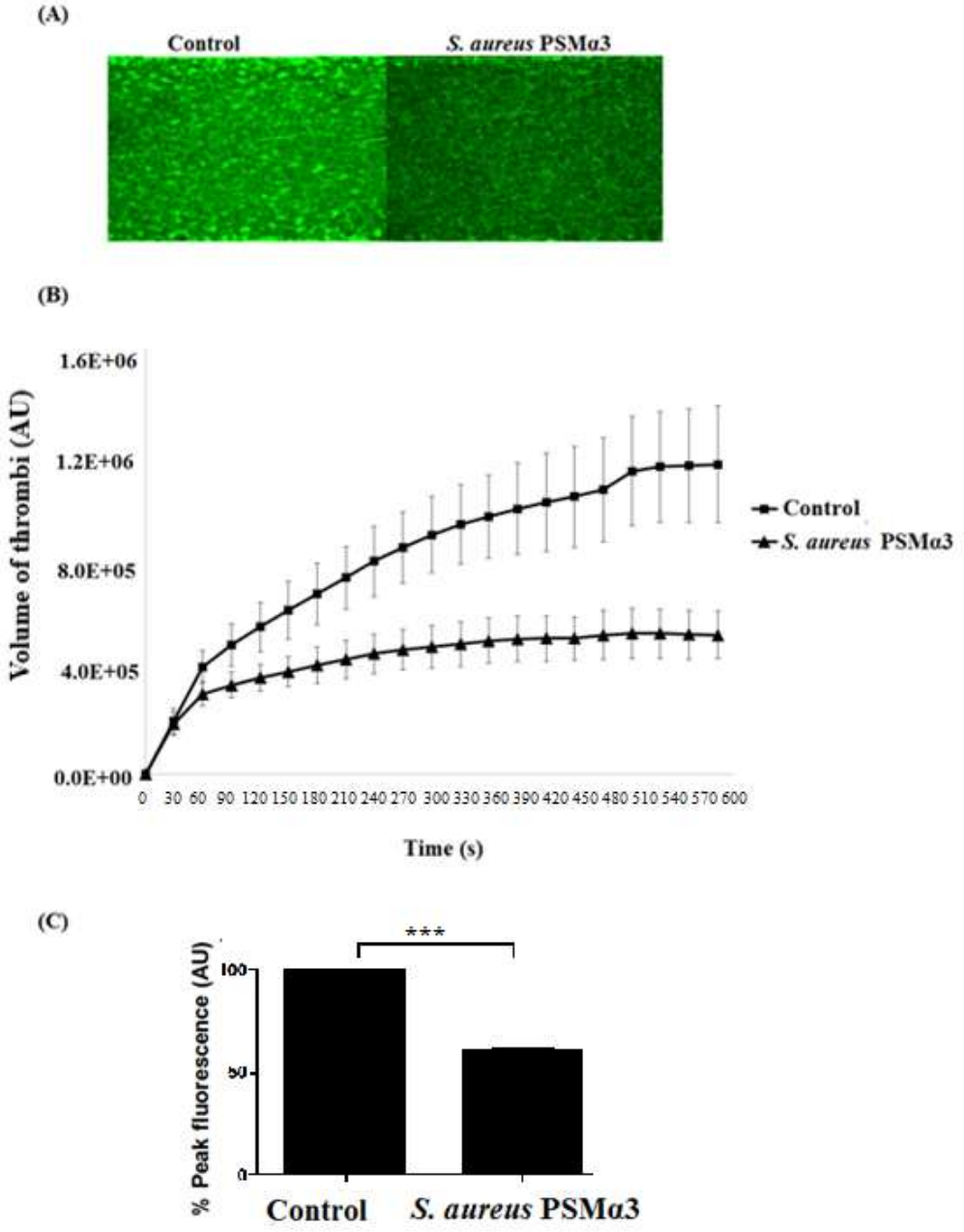


Figure 3.6: *S. aureus* PSMα3 inhibits formation of thrombus. (A) Platelets within whole human blood were pre-incubated with 3,3 dihexyloxacarbocyanine iodide and treated with Tyrode's buffer as control. The labelled blood was the flowed over a collagen-coated (400 µg/mL) slide at a shear rate of 20 dynes/cm^{-2} for 10 minutes. The images of thrombi formation in this assay were recorded and captured every 30 seconds over 10 minutes by Nikon eclipse (TE 2000-U) microscope. **(B)** Volume of thrombi formed. **(C)** Slidebook™ 5 software was used to calculate the percent peak fluorescence intensity. (n=3; Student's t-test, ***P<0.001).

3.2.3 Inhibition of integrin $\alpha_{IIb}\beta_3$ activation and granules release by *S. aureus* PSMα3

3.2.3.1 Effect of *S. aureus* PSMα3 on upregulation of affinity of integrin $\alpha_{IIb}\beta_3$

S. aureus PSMα3 has an inhibitory effect on platelet activation. In order to check its effect on activation of integrin $\alpha_{IIb}\beta_3$, fibrinogen binding was used as a marker for the integrin's activation. FITC-labelled rabbit anti-human fibrinogen antibody (Table 2.14) was used to detect fibrinogen in response to different concentrations of PSMα3.

PRP was collected and prepared as described in section 2.6 and incubated with increasing concentrations of *S. aureus* PSMα3. The negative control in this experiment was untreated platelets, labelled with FITC-labelled rabbit anti-human fibrinogen antibody without adding any platelet agonists, and the positive controls were 0.05 U/mL of thrombin and 0.5 µg/mL of CRP-XL. The median fluorescence intensity of fibrinogen binding of samples was measured by flow cytometry (Model C6, BD Accuri Cytometers, USA) as described in section 2.9.

Data showed that fibrinogen binding to platelets was reduced by *S. aureus* PSMα3 in a dose-dependent manner (Figure 3.7 and Figure 3.9). Thrombin (0.05 U/mL)–induced fibrinogen binding was inhibited at all concentrations of *S. aureus* PSMα3 tested (3.8, 7.7, 15.38 and 30.7 µM) by ~66% inhibition of fibrinogen binding with PSMα3 (30.7µM) (median fluorescence intensity is shown in Figure 3.7A). Similarly, inhibition of fibrinogen binding mediated by CRP-XL (0.5 µg/mL) was also observed at all PSMα3 concentrations (3.8, 7.7, 15.38 and 30.7 µM; median fluorescence intensity is shown in Figure 3.7B). These data were found to be consistent with inhibition of platelet aggregation caused by *S. aureus* PSMα3 as shown in section 4.2.1.

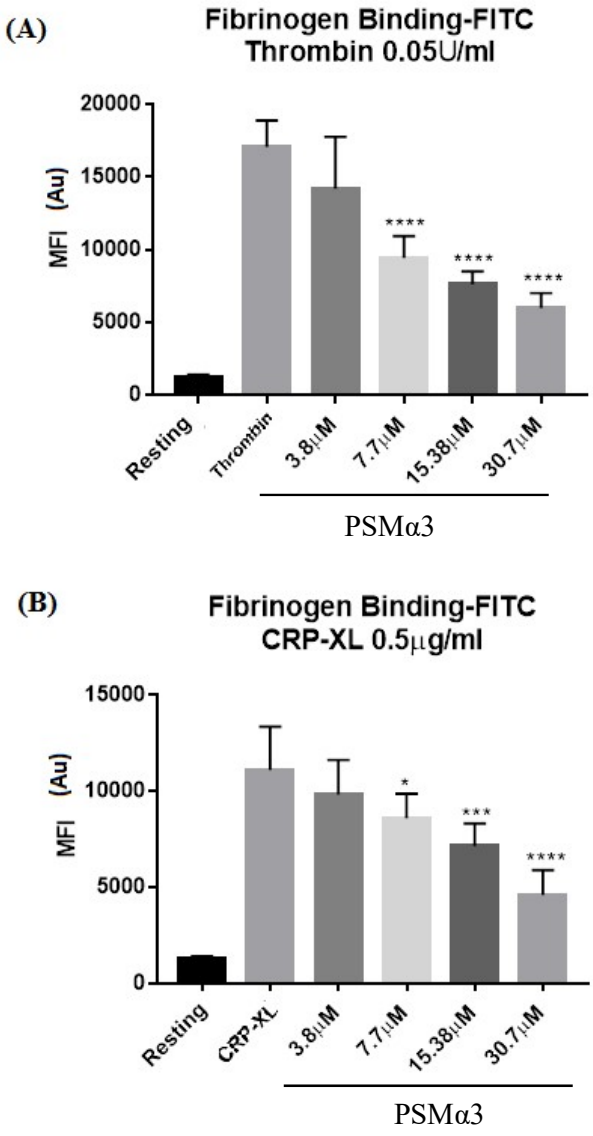


Figure 3.7: *S. aureus* PSMα3 inhibits fibrinogen binding to platelets. Human PRP was incubated with *S. aureus* PSMα3 (3.8, 7.7, 15.38, 30.7 μM) and then stimulated by **(A)** thrombin (0.05 U/mL) or **(B)** CRP-XL (0.5 μg/mL). The median fluorescence intensity (MFI) of fibrinogen binding in samples was measured by flow cytometry. The result showed that fibrinogen binding was inhibited by *S. aureus* PSMα3 in a dose-dependent manner. This inhibition of fibrinogen binding means that *S. aureus* PSMα3 prevents activation of integrin $\alpha_{IIb}\beta_3$. These data represent the ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test,*P≤0.05, ***P≤0.001, ****P≤0.0001).

Platelet aggregation was inhibited by *S. aureus* PSMα3 and data indicate that this occurs prior to development of platelet aggregation. Reduced binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ would hinder the stabilisation of platelet aggregates. The level of fibrinogen binding to $\alpha_{IIb}\beta_3$ in response to agonist was studied to explore the impact of *S. aureus* PSMα3 on upregulation of integrin. These data were in agreement with the reduction observed by PSMα3 over aggregation in response to thrombin and CRP-XL. Also this demonstrated that *S. aureus* PSMα3 was not affected by plasma proteins.

3.2.3.2 Effect of *S. aureus* PSMα3 on the exposure of P-selectin on human platelets

P-selectin has a lectin domain which presents on the surface of platelets during activation (Tedder *et al.*, 1995). P-selectin binds to other lectin domains on other nearby platelets, causing stabilisation of platelet aggregates. According to the pervious results *S. aureus* PSMα3 inhibits binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$ (3.2.3.1), so this experiment was setup to see if there was an effect of *S. aureus* PSMα3 on platelet granules secretion.

P-selectin is considered as a classical marker for the release of α granules from platelets (McEver, 2002). PSMα3 was tested for its effect on the release of platelet α granules. PE-Cy5 anti-human CD62P antibody (Table 2.14) was used to detect P-selectin exposure on the surface of human platelets.

Human PRP was incubated with different concentrations of *S. aureus* PSMα3. In the case of thrombin, 25 μg/mL of GPRP was added for prevent fibrin polymerisation. The median fluorescence intensity of samples was measured by flow cytometry (Model C6, BD Accuri Cytometers, USA) as described in section 2.9.

In this experiment, resting platelets were used as negative controls, while platelets treated with 0.5 µg/mL of CRP-XL or 0.05 U/mL of thrombin were used as positive controls.

PSMα3 inhibited thrombin (0.05 U/mL)-induced P-selectin exposure by in a dose-dependent manner (Figure 3.8A). In comparison with the control (positive control; Figure 3.8A), PSMα3 inhibited the exposure of P-selectin by 60% and 80% at 15.38 and 30.7µM of PSMα3, respectively (Figure 3.8A). Similarly, exposure of P-selectin by CRP-XL (0.5 µg/mL) was also inhibited at all concentrations of *S. aureus* PSMα3 tested (3.8, 7.7, 15.38 and 30.7µM) (Figure 3.8B). Maximal inhibition of ~73% was obtained with 30.7µM of PSMα3 cf the positive control (Figure 3.8B).

To conclude, *S. aureus* PSMα3 in a dose-dependent manner caused reduction of the exposure of P-selectin after stimulation by thrombin or CRP-XL (Figure 3.8 and Figure 3.9). The exposure and release of P-selectin from α granules of the platelet in response to thrombin and CRP-XL was inhibited by *S. aureus* PSMα3, a result which was consistent with inhibition of fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ (3.2.3.1).

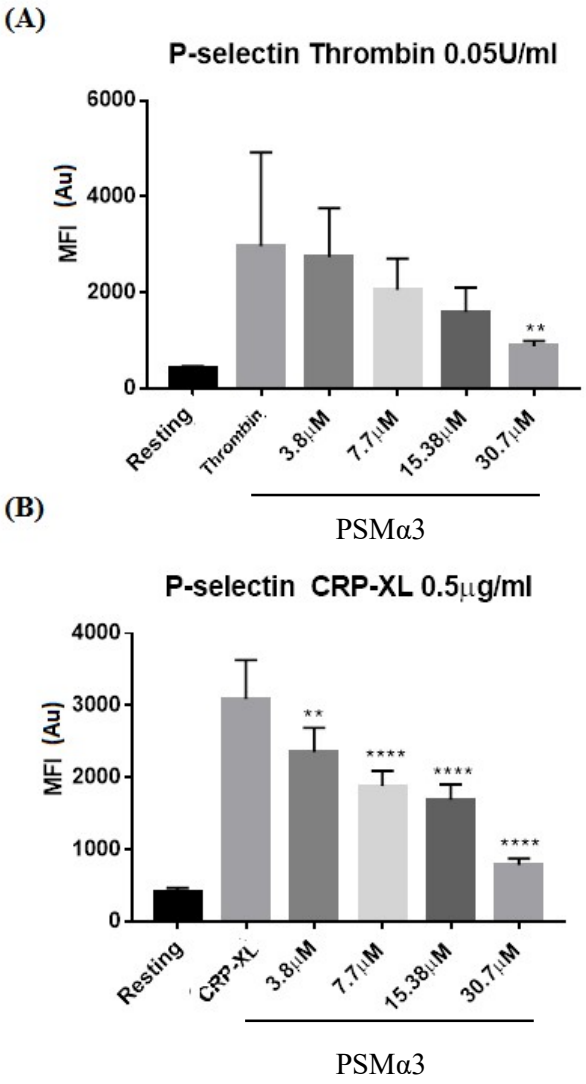


Figure 3.8: *S. aureus* PSMα3 inhibits the expression of P-selectin on platelets. Human PRP was incubated with *S. aureus* PSMα3 and then stimulated by **(A)** thrombin (0.05 U/mL) or **(B)** CRP-XL (0.5 µg/mL). The median fluorescence intensity (MFI) of exposure of P-selectin in samples was measured by flow cytometry. The result showed that exposure of P-selectin was inhibited by *S. aureus* PSMα3 in a dose-dependent manner. This inhibition of P-selectin means that *S. aureus* PSMα3 prevents the release of α granules of platelets. These data represent ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, **P≤0.01, ****P≤0.0001).

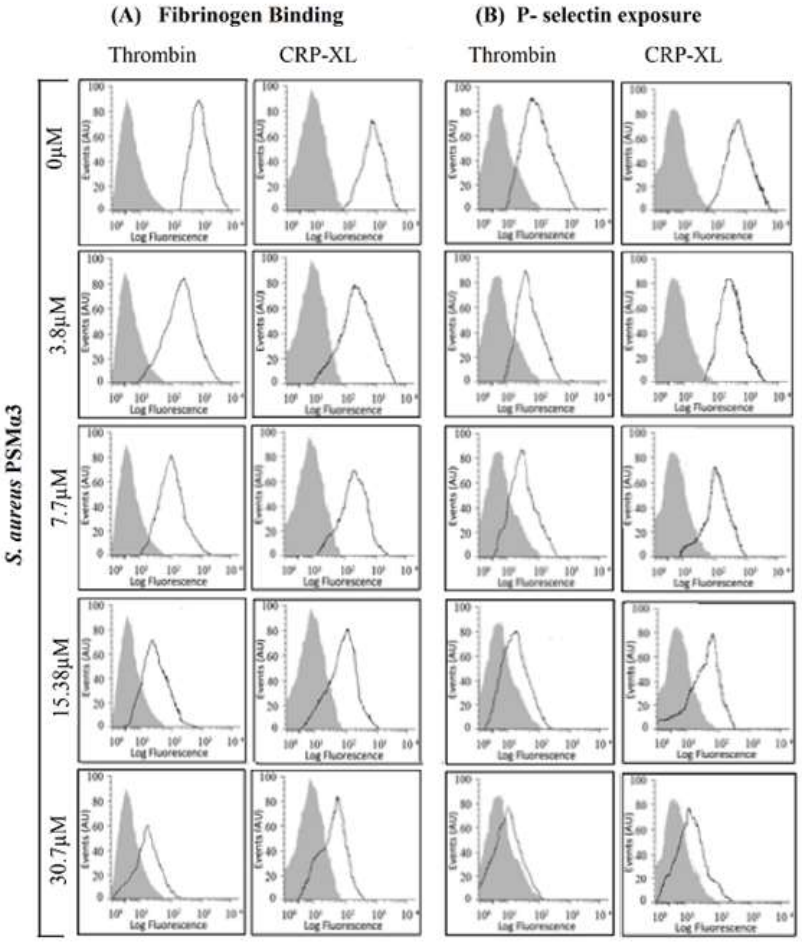


Figure 3.9: **Histograms representing a typical experiment of inhibition of fibrinogen binding (A) or P-selectin exposure (B)** in presence of thrombin (0.05 U/mL) or CRP-XL (0.5 µg/mL). Grey histogram shows control non-stimulated platelets.

3.2.4 *S. aureus* PSMα3 affects outside-in signalling of platelet integrin $\alpha_{IIb}\beta_3$

Outside-in signalling of integrin $\alpha_{IIb}\beta_3$ is an important secondary activation pathway downstream of platelet agonists (Shattil *et al.*, 1998). Outside-in signalling is initiated following fibrinogen binding and clustering of integrin $\alpha_{IIb}\beta_3$ (Shattil *et al.*, 1998). Initiation of outside-in signalling leads to shape change of platelets and spreading (Shattil *et al.*, 1998). To determine whether the *S. aureus* PSMα3 has an effect on upregulation of outside-in signalling, clot retraction and platelet spreading were analysed in platelets treated with *S. aureus* PSMα3.

3.2.4.1 Inhibition of clot retraction by *S. aureus* PSMα3

S. aureus PSMα3 reduced fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ (3.2.3.1). Therefore studying the effect of *S. aureus* PSMα3 on outside-in signalling of integrin $\alpha_{IIb}\beta_3$ is important. Data showed the clot weight in the *S. aureus* PSMα3 was 3-fold higher when cf control (Figure 3.10B). The experiment showed that *S. aureus* PSMα3 has an effect on outside-in signalling.

Thrombin was added to PRP to initiate clots in the presence or absence of *S. aureus* PSMα3 (15.38 and 30.7μM), and clot formation was monitored for 1 hour (Figure 3.10A) by measuring remaining clot weight (Figure 3.10B). Clot retraction was inhibited in the presence of PSMα3 (15.38 and 30.7μM) at 1 hour compared with the control (Figure 3.10A). Data showed the clot weight in the *S. aureus* PSMα3 was 3-fold higher when cf control (Figure 3.10B). This suggests that *S. aureus* PSMα3 has a strong impact on outside-in signalling of integrin $\alpha_{IIb}\beta_3$. This result is consistent with inhibition of integrin $\alpha_{IIb}\beta_3$ activation (3.2.3.1).

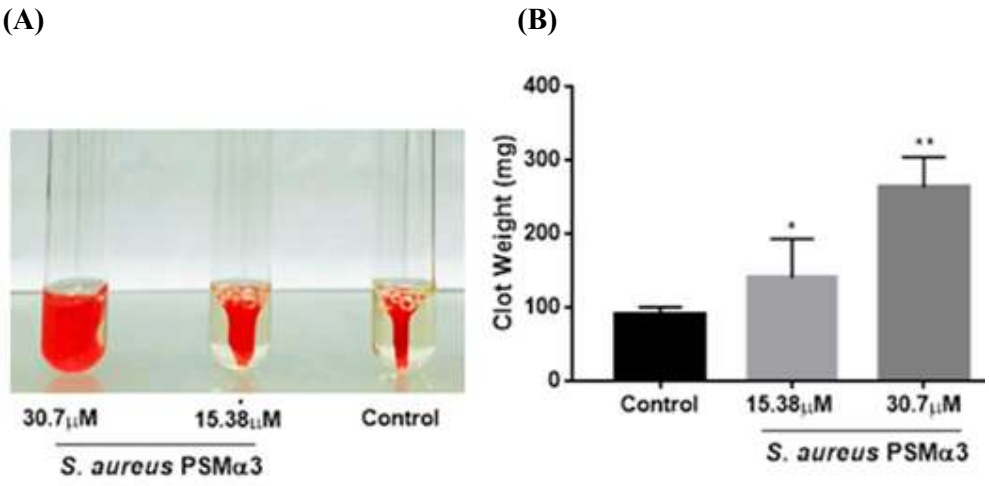


Figure 3.10: Inhibition of the clot retraction by *S. aureus* PSMα3. PRP (200 µL) and 5 µL of red blood cells were treated with 15.38 or 30.7 µM of *S. aureus* PSMα3 and pre-incubated for 5 minutes at room temperature. After that, 1 U/mL of thrombin was used to generate the clot formation. **(A)** Photographs of clots after 1 hour. **(B)** Cumulative data calculating clot weight. (n=3; one-way ANOVA with Tukey as a post hoc test, *P≤0.05, **P≤0.01)**.**

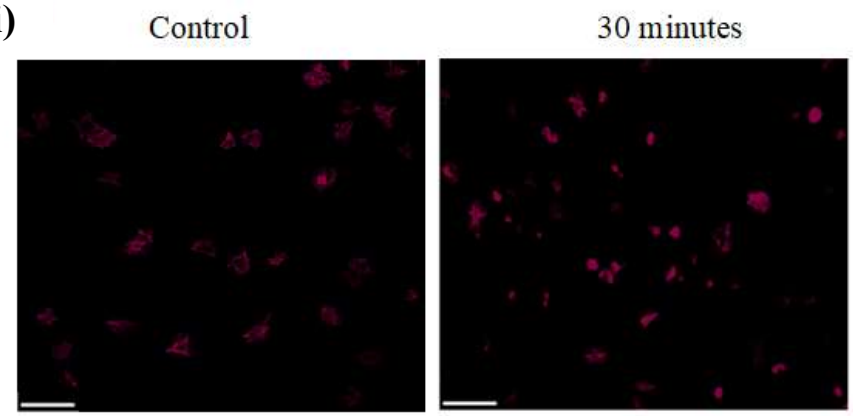
3.2.4.2 *S. aureus* PSMα3 inhibits platelet spreading on fibrinogen

According to the previous result, PSMα3 inhibits clot retraction, so platelet spreading on immobilised fibrinogen was studied as a marker for outside-in signalling of integrin $\alpha_{IIb}\beta_3$. Platelet spreading is distinctly regulated by outside-in signalling that causes shape change of platelets at the site of injury (Lee *et al.*, 2012).

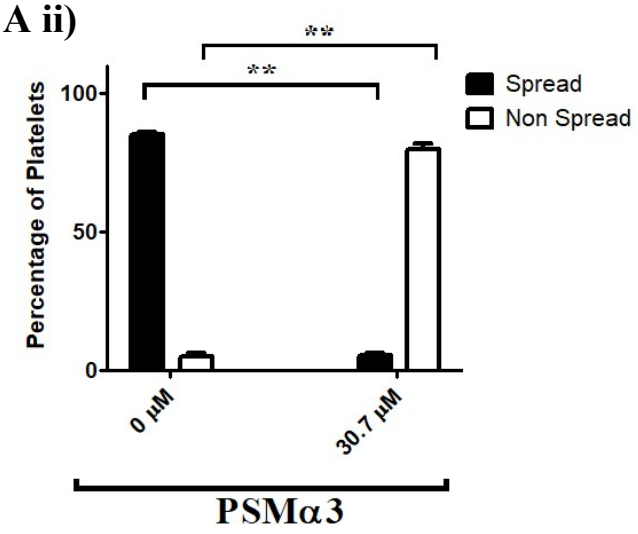
In this experiment, *S. aureus* PSMα3 was investigated for its ability to inhibit platelet spreading on immobilised fibrinogen. Platelets were incubated with *S. aureus* PSMα3 based on a time course of 30, 45 and 60 minutes. Comparison between controls and platelets treated with *S. aureus* PSMα3 was done based on four features of platelets; (1) formation of filopodia, (2) lamellipodia, (3) spreading or (4) non-spreading of platelets.

These data showed that *S. aureus* PSMα3 (30.7 μM) inhibits platelet spreading on immobilised fibrinogen when cf controls that were carried out to different end-points, 30, 45 and 60 minutes (Figure 3.11, A i, B i, C i). *S. aureus* PSMα3 caused a significant reduction in the rate of spreading (Figure 3.11, A ii, B ii, C ii) and formation of filopodia and lamellipodia when cf controls (Figure 3.11, A iii, B iii, C iii). PSMα3 caused ~70% inhibition of platelet spreading and fewer platelets were able to generate filopodia and lamellipodia compared with their controls at 30 minutes (Figure 3.11, Aii and Aiii). Inhibition of platelet spreading by PSMα3 was also observed over longer time-courses for spreading experiments. The toxin also caused a significant inhibition in the rate of spreading and formation of filopodia and lamellipodia at 45 minutes (Figure 3.11, B ii and B iii) and at 60 minutes when cf their controls (Figure 3.11, C ii and C iii). These data were found to be consistent with inhibition of integrin $\alpha_{IIb}\beta_3$ activation and inhibition of the clot retraction by *S. aureus* PSMα3 (section 3.2.3.1 and 3.2.4.1).

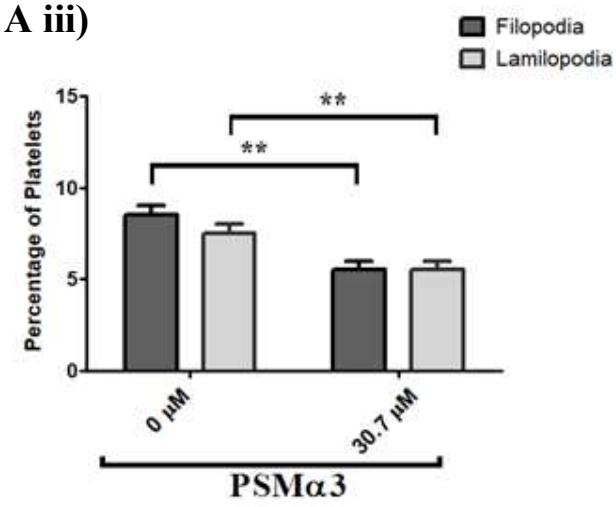
A i)



A ii)



A iii)



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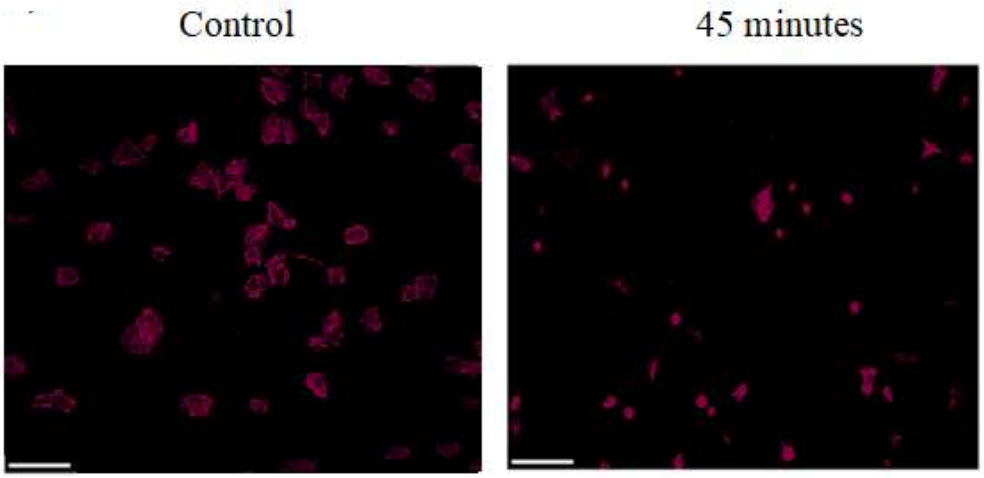
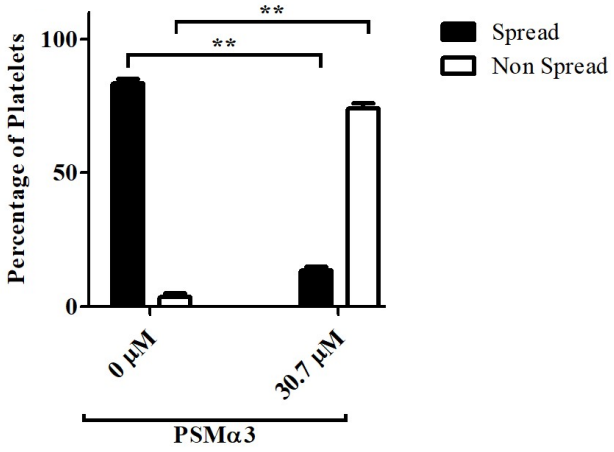
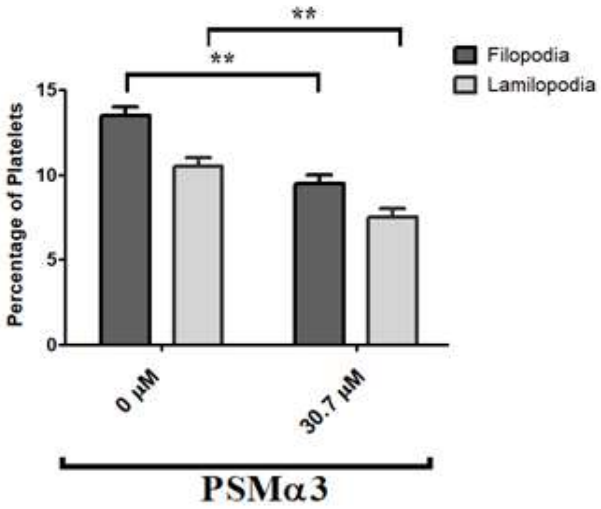
B i)



B ii)



B iii)



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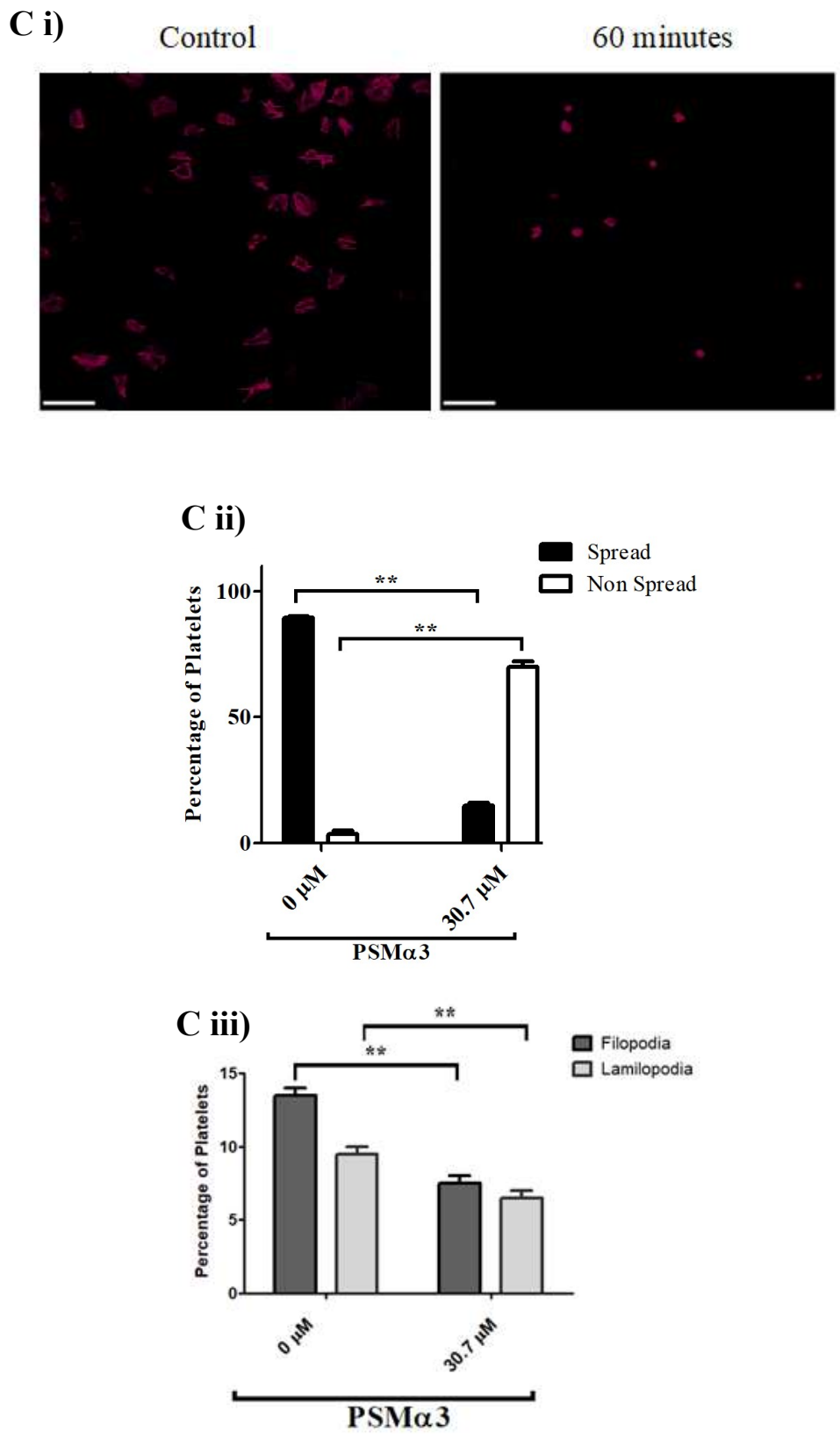


Figure 3.11: *S. aureus* PSMα3 inhibits platelet spreading on fibrinogen. (A i ,B i, C i) Representative images of platelet spreading on fibrinogen. Phalloidin Alexa-488 was

used for staining platelets. Oil immersion with 100x lens was used for visualisation of platelets treated with *S. aureus* PSMα3 (30.7 μM). (Scale bars – 100μm) (n=3). The rate of spreading and formation of filopodia and lamellipodia in platelets treated with *S. aureus* PSMα3. Data plotted as percentage of platelets, spread and non-spread after incubating platelets with *S. aureus* PSMα3 (30.7 μM) for 30 minutes **(A ii)**, 45 minutes **(B ii)** and 60 minutes **(C ii)**. Platelets with filopodia and platelets with lamellipodia after incubating platelets with *S. aureus* PSMα3 (30.7 μM) for 30 minutes **(A iii)**, 45 minutes **(B iii)** and 60 minutes **(C iii)**. Data represent mean values ± SEM. (n=3; Student's t-test, **P<0.01).

3.3 Conclusions

S. aureus PSMα3 causes inhibition of platelet activation *in vitro* (Figures, 3.2-3.6). Platelet aggregation mediated by thrombin or CRP-XL, two agonists with distinct activation pathways, was inhibited in the presence of *S. aureus* PSMα3 (3.2.2.1). This indicates that *S. aureus* PSMα3 prevents primary signalling events of thrombin or CRP-XL pathways. The effect of *S. aureus* PSMα3 was also tested in conditions analogous to the human bloodstream. *S. aureus* PSMα3 mediated inhibition was not affected by shear forces that have an important physiological role for the interaction of platelet receptors and their ligands.

S. aureus PSMα3 downregulated integrin $\alpha_{IIb}\beta_3$ binding to fibrinogen (Figure 3.7), which is important for supporting platelet aggregation. This indicates that *S. aureus* PSMα3 controls inside-out signalling of integrin $\alpha_{IIb}\beta_3$ by an elusive mechanism.

P-selectin is important for recruitment and activation of nearby platelets. Exposure of P-selectin on the surface of platelets was also reduced (Figure 3.8), indicating that *S. aureus* PSMα3 prevents the secretion of α-granules from platelets. Preventing α-granules release from platelets causes failure to stabilise aggregates and recruit other platelets.

Clot retraction and platelet spreading assays were carried out as markers for outside-in signalling of integrin $\alpha_{IIb}\beta_3$. The outside-in signalling was reduced in platelets treated with *S. aureus* PSMα3 (Figure 3.10 and 3.11) as shown in section 3.2.4. In summary, *S. aureus* PSMα3 was found to be a powerful inhibitor of platelet activation.

Finally, one more experiment should be conducted if these studies are to be repeated. A scrambled peptide would be a good negative control for these studies to confirm that all of the effects reported here are specific to PSMα3 and not just the presence of exogenous

peptide. A scrambled peptide would exclude any false positive results. Design of a scrambled peptide can done using minotopes, a web-based tool, (http://www. Minotopes.com/peptideLibraryScreening.asp).

CHAPTER 4

Effect of *S. aureus* PSMα3 on human platelets is independent of receptor FPR2

4. Effect of *S. aureus* PSMα3 on human platelets is independent of receptor FPR2

4.1 Introduction

S. aureus can secrete a set of formylated peptides that have the ability to both activate and modulate the chemotaxis of neutrophils (Durr *et al.*, 2006). All *de novo* bacterial proteins contain an N-formyl methionine (Adams *et al.,* 1966), which is commonly removed during secretion (Mazel *et al.,* 1994). However, the presence of this N-formyl methionine is detected by the host's innate immune system as it is a signal for the presence of bacterial infection (Fu *et al.*, 2006). For example, PSMα3 toxin has an N-formyl methionine (Wang *et al.*, 2007) that is detected by neutrophils via FPR2 (Kretschmer *et al.*, 2010). FPR2 belongs to a family of formyl peptide receptors (FPRs); FPR1, FPR2 and FPR3. FPRs are found in different cells such as leukocytes, endothelial cells, platelets and astrocytes (Fu *et al.*, 2006; Migeotte *et al.*, 2006). The N-formyl group found on bacterial peptides is key for the activation of FPRs (Schiffmann *et al.*, 1975; Tempel *et al.*, 1970).

The aim of the work in this chapter was to evaluate the role of FPR2 in response to *S. aureus* PSMα3. Experiments in this chapter were focused on the identification and localisation of FPR2 in human platelets. Additionally, work was carried out to investigate the action of *S. aureus* PSMα3 on human platelets and how this is mediated, through the identification of the cognate receptor.

4.2 Results

4.2.1 *S. aureus* PSMα3 works independently of its receptor FPR2 in human platelets

4.2.1.1 Expression and localisation of FPR2 in human platelets

Czapiga and colleagues (2005) reported expression of the *FPR2* gene in megakaryocytes. Thus, the main goal of this experiment was to confirm the expression of the FPR2 protein in human platelets.

Proteins were collected from platelet lysates ($4x10^8$ cells/mL) of two human donors as described in section 2.5. These proteins were resolved on a 12% (w/v) SDS-PAGE gel and transferred to PVDF membrane as described in section 2.15.2. Anti-FPR2 antibody (Table 2.16) was used to detect the presence of FPR2 by Western immunoblotting. Rabbit anti-human 14-3-3ζ obtained from Santa Cruz Biotechnology, USA, was used to detect the 14-3-3ζ. This protein was detected in each sample as a loading control to ensure loading proteins have equal levels on Western immunoblots.

The result showed a band corresponding to the size of FPR2 is present in human platelets (Figure 4.1). The molecular weight of the FPR2 is 38 kDa without posttranslational modifications (Ho *et al.*, 2018). This result confirms the expression of the FPR protein in human platelets.

To determine the location of FPR2 in human platelets, immunocytochemistry was used as described in section 2.13. Anti-GPIb AlexaFluor-488-labeled was used as a maker for the platelet surface. The location of FPR2 was determined by anti-FPR2 AlexaFluor

647-labeled. The immunofluorescence data showed that FPR2 exists on the surface of the platelets with a punctate arrangement (Figure 4.2).

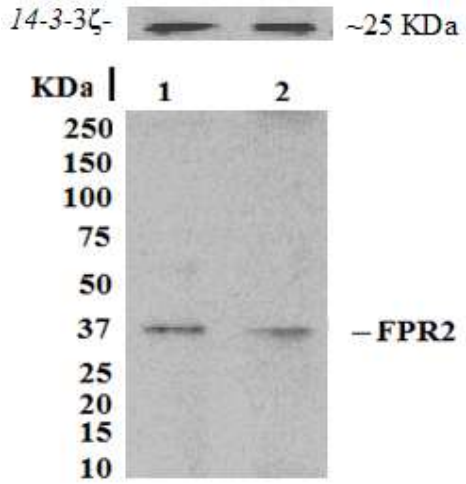


Figure 4.1: Presence of the FPR2 in human platelets. Expression of FPR2 in two different human platelet lysates (Lane 1, Lane 2). Rabbit anti-FPR2 antibody was used for detecting FPR2 expression in this western blot. The protein14-3-3ζ was measured as a loading control by G: BOX Chemi. (n=3).

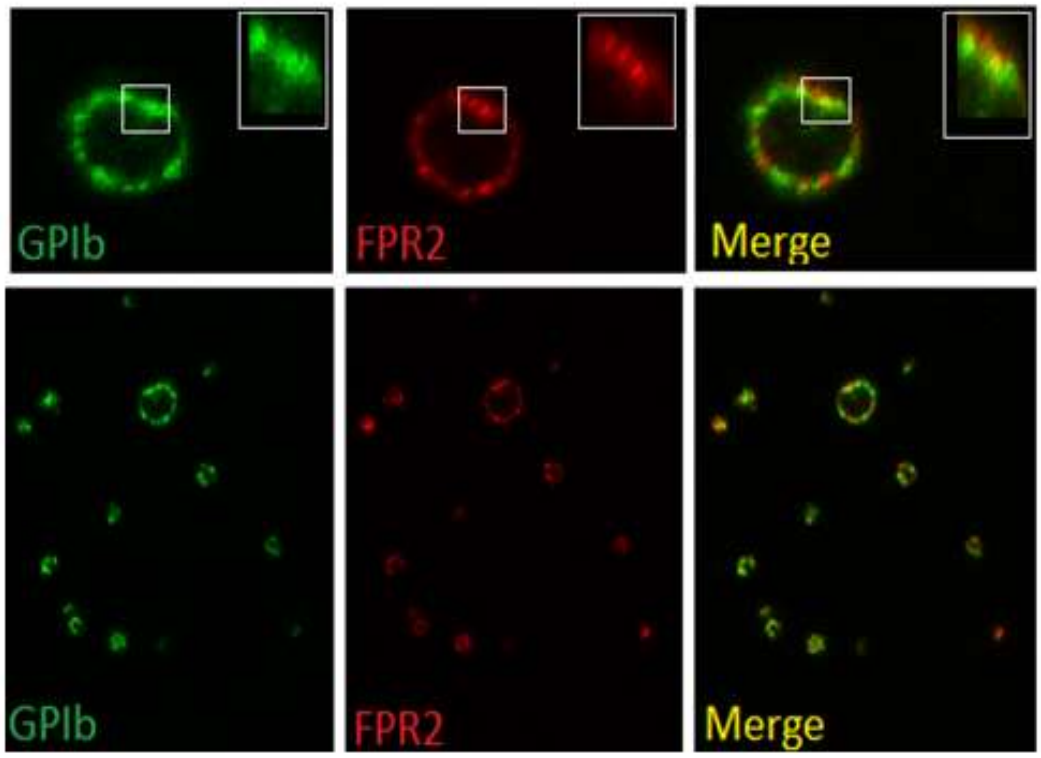


Figure 4.2: Localisation of FPR2 on the surface of platelets. Immunocytochemical studies were carried out with PRP fixed with 4% paraformaldehyde. Anti-GPIb AlexaFluor-488-labeled and anti-FPR2 AlexaFluor 647-labeled were used in this study. A confocal microscopy with oil immersion 100x lens was used for the visualisation of this localisation of FPR2 in platelets. This study confirmed the surface localisation of FPR2 in human platelets (n=3).

4.2.1.2 Investigation of the presence of a functional FPR2 on platelets

To determine whether FPR2 on platelets is functional or not, platelets were stimulated with an FPR2-specific agonist, MMK-1 (Hu *et al.*, 2001; Quehenberger *et al.*, 1993) and Trp-Arg-Trp-Trp-Trp-Trp-CONH(2) (WRWWWW (WRW(4))), a selective FPR2 antagonist (Bae *et al.*, 2004).

This experiment was done with washed human platelets ($4x10^8$ cells/mL), as described in section 2.5, to measure the effect of MMK-1 (Table 2.17) on human platelet aggregation, as described in section 2.7. Thrombin (Table 2.17) was used as a positive control for platelet aggregation and the negative control was platelets treated with Tyrode's buffer.

Data showed that MMK-1 causes platelet aggregation (Figure 4.3) and WRW4 (5 μM) is able to prevent MMK-1- induced platelet aggregation (Figure 4.4A). These data demonstrate that FPR2 is a functional receptor in platelets. Moreover, data showed that WRW4 decreased the response to MMK-1, indicating that MMK-1 works dependent on FPR2. However, WRW4 (5 μM) did not abrogate the action of *S. aureus* PSMα3 (Figure 4.4B), suggesting that *S. aureus* PSMα3 works independently of FPR2. To conclude, this effect of *S. aureus* PSMα3 on platelets was not meditated by FPR2. Therefore, the action of *S. aureus* PSMα3 is due to binding to another platelet receptor (Figure 4.4).

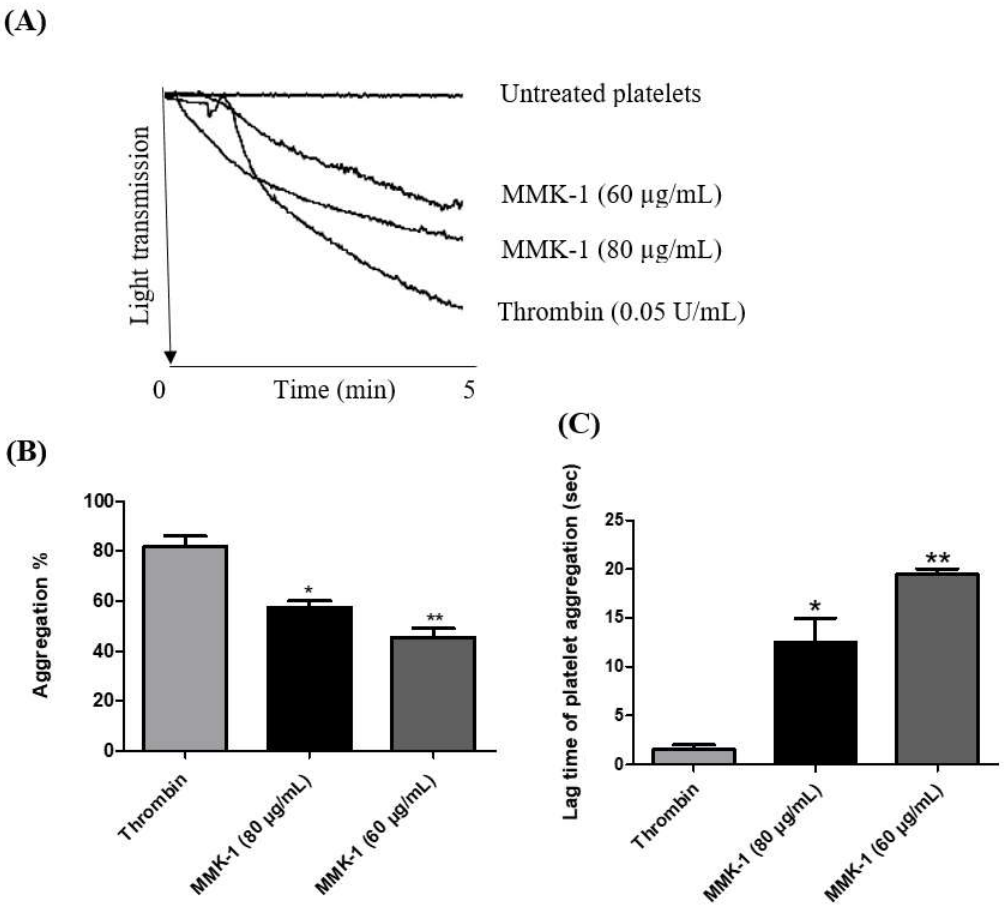


Figure 4.3: Effect of MMK-1 on platelet aggregation. (A) Representative traces. Washed human platelets ($4x10^8$ cells/mL) were treated with two concentrations of the MMK-1 and recorded by a Chronolog 490-2d aggregometer for 5 minutes at an optical density (OD) of 405 nm. **(B)** Percentage of platelet aggregation in response to MMK-1. MMK-1 (80 µg/mL) showed an ability to activate the aggregatory response of platelets. **(C)** The lag time of platelet aggregation in response to MMK-1. High concentration of MMK-1 has a short time to cause platelet aggregation when compared to MMK-1 60 µg/mL. These data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, *P≤0.05, **P≤0.01).

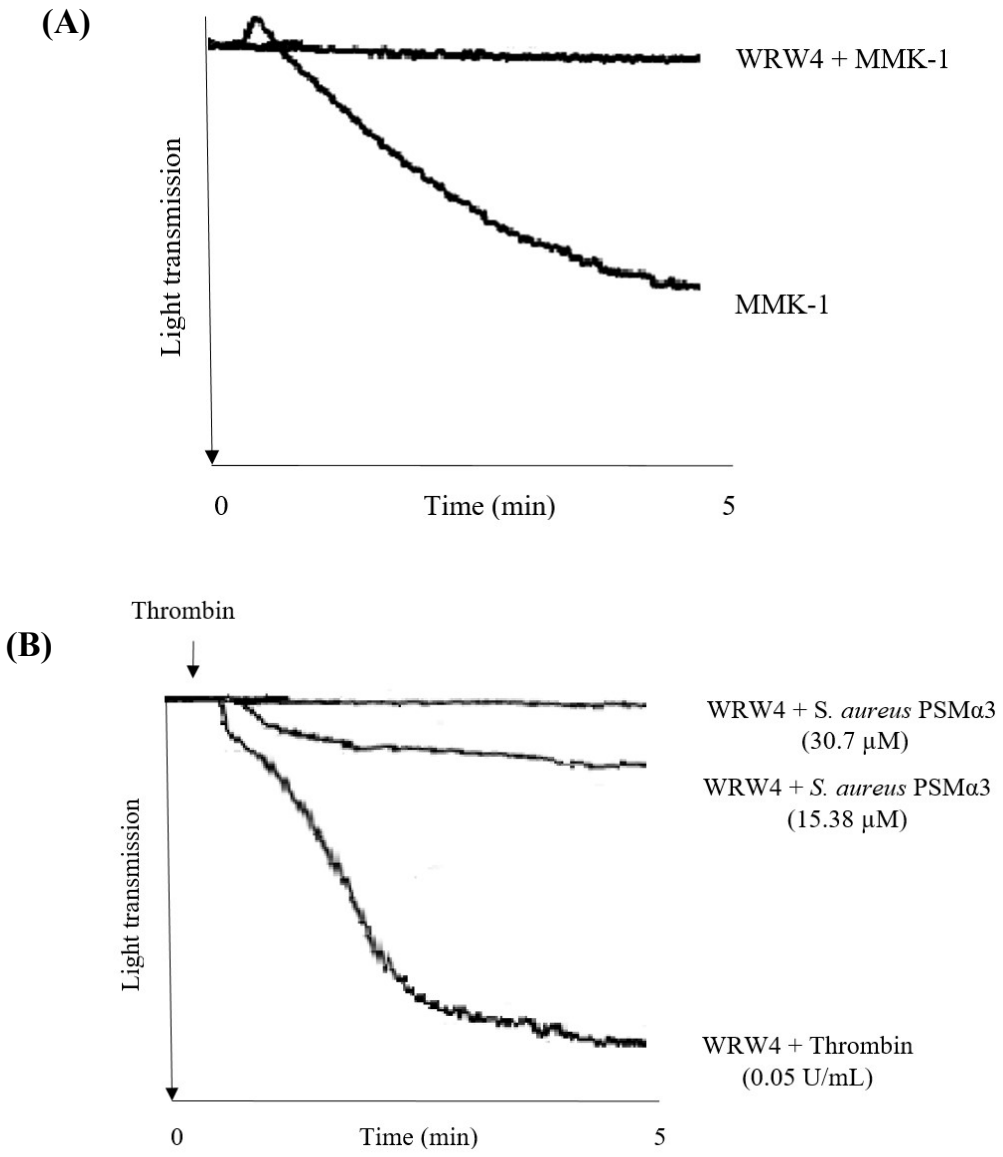


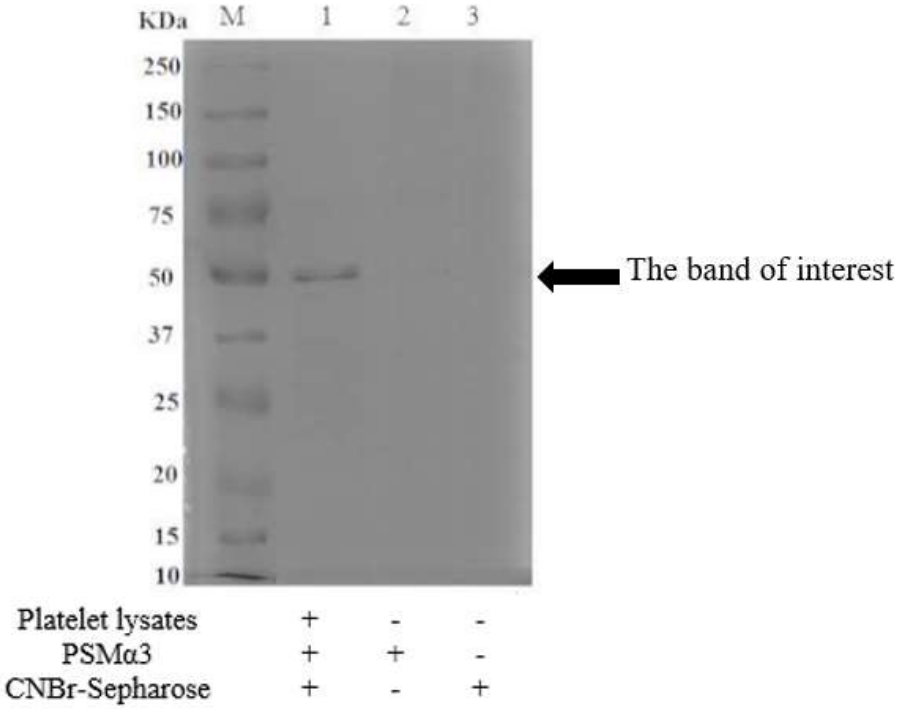
Figure 4.4: Effect of WRW4 on actions of MMK-1 and PSMα3. Representative trace. Washed human platelets ($4x10^8$ cells/mL) aggregation was recorded *via* chronolog 490-2d aggregometer for 5 minutes. **(A)** Platelets were pre-incubated with WRW4 (5 µM) for 10 minutes, MMK-1 (80 µg/mL) was added then recorded for 5 minutes. Platelets were stimulated by MMK-1 (80 µg/mL) and recorded for 5 minutes. **(B)** Platelets were pre-incubated with WRW4 for 10 minutes then, *S. aureus* PSMα3 (15.38 and 30.7µM) was added then incubated for 5 minutes. Platelets were then stimulated with thrombin (0.05 U/mL). Aggregation was recorded for 5 minutes. (n=3).

4.2.2 Determination of platelet protein that involved in interaction with *S. aureus* PSMα3

As a consequence of the previous data, further experiments were conducted to identify the platelet receptor for *S. aureus* PSMα3. A co-precipitation assay as described in section 2.18, was carried out with *S. aureus* PSMα3 coupled to CNBr-Sepharose, as in described section 2.17. Proteins from platelet lysates were treated with *S. aureus* PSMα3 coupled to CNBr-Sepharose. Eluates were run on a 12% SDS-PAGE gel and silver stained (Table 2.16). The band of interest (Figure 4.5A, Lane 1) was cut out of the gel, as described in 2.19.1, and submitted for analysis by mass spectrometry as described in section 2.19.2. PSMα3 without platelets or beads (Figure 4.5A, Lane 2) and beads without any PSMα3 (Figure 4.5A, Lane 3) were used as controls.

The result of mass spectrometry analysis for identifying proteins has provided with score and coverage values. The former, which is an estimate of how well the mass spectrum matches the expected spectrum for the particular identified protein. The latter, which is a percentage of the protein sequence covered by the identified peptides. Therefore, high score and coverage values provide high confidence about the identification of the protein. The result of the mass spectrometry analysis showed that VPAC1 is likely to be the major surface protein that co-precipitated with *S. aureus* PSMα3 (Figure 4.5B). This result was selected by high score and coverage values of the mass spectrometry analysis (Figure 4.5B). This high score of VPAC1 suggests that it is highly likely to be a major platelet surface receptor for *S. aureus* PSMα3. Other platelet proteins like GPIbβ and Guanine nucleotide-binding protein G(t) subunit alpha-1, were found in the mass spectrometry analysis (Figure 4.5B). But, low coverage and score values suggest that they might be co-precipitating with some other receptor (such as VPAC1) with higher affinity for PSMα3.

(A)



(B)

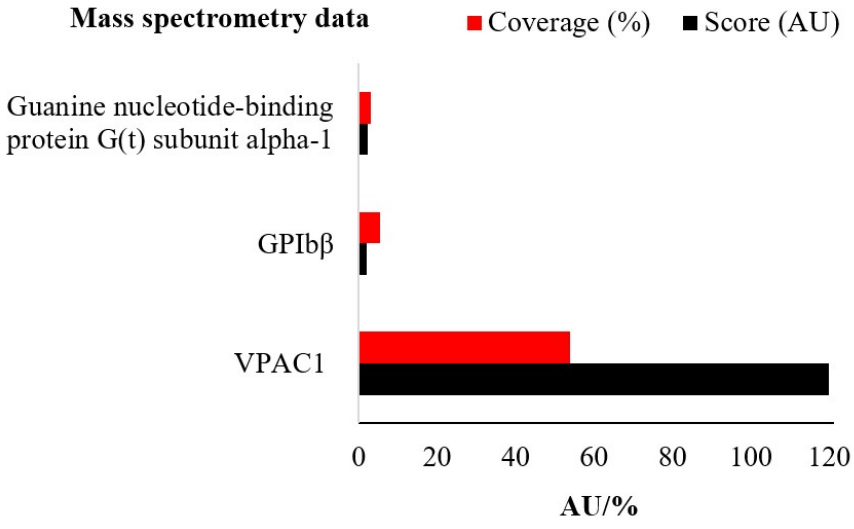


Figure 4.5: Co-precipitation assay result. (A) Silver stained SDS PAGE gel showed a band of platelet proteins co-precipitated with *S. aureus* PSMα3. The band (Lane 1) was cut out and analysed by Orbitrap Mass spectrometer. PSMα3 without platelets or beads (Lane 2) and beads without any PSMα3 (Lane 3) were used as controls. **(B)** Mass spectrometry result showed that VPAC1 receptor is likely as the major platelet surface that co-precipitated with *S. aureus* PSMα3.

A completion assay was carried out to study the effect of a VPAC1 inhibitor on the action of *S. aureus* PSMα3 (Figure 4.6-4.7). Stimulation of platelets with VIP (15 μM), a selective VPAC1 receptor agonist, caused phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Serine-157. VASP is a protein that belongs to the family of Ena/VASP and is highly expressed in human platelets (Eigenthaler *et al.*, 1992; Halbrugge *et al.*, 1989). VASP plays a role in filament assembly and organization of actin (Harbeck *et al.*, 2000) and is a common substrate for PKA and PKG in human platelets (Waldmann *et al.*, 1987). VASP phosphorylates at Ser^{157} and Ser^{239} by PKA and PKG, respectively (Butt *et al.*, 1994). Phosphorylation at these residues leads to reduced ability of VASP to interact with actin (Harbeck *et al.*, 2000). Therefore, phosphorylation of VASP correlates with inhibition of platelet aggregation (Horstrup *et al.*, 1994). Activation of VPAC1 reduces platelet aggregation (Freson *et al.*, 2004).

Platelets were incubated with *S. aureus* PSMα3, VIP (15 μM) or PGI_2 for 5 minutes. Thrombin (0.05 U/mL) was added to platelets and incubated for 90 seconds as a negative control. VASP phosphorylation was detected by western immunoblot with anti-VASP antibody. The result showed that VASP phosphorylation was at residue Ser157 in platelets treated with PSMα3, VIP (Figure 4.6A, Lane 2-3) or PGI_2 (Figure 4.6A, Lane 5), whereas platelets stimulated with thrombin (0.05 U/mL) did not show any signs of VASP phosphorylation (Figure 4.6A, Lane 1).

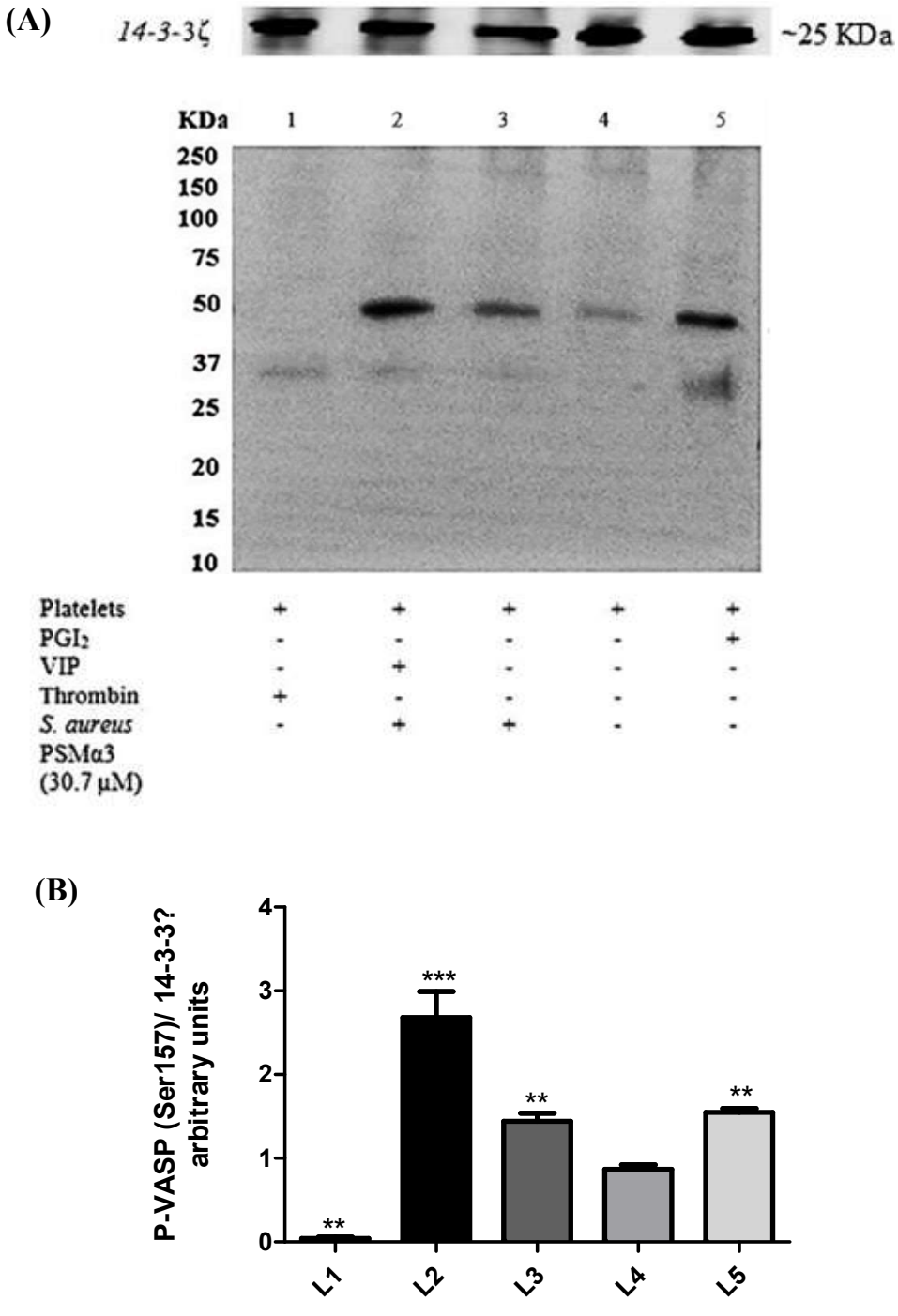


Figure 4.6: Effect of VPAC1 receptor agonist on the action of *S. aureus* PSMα3. (A) Western blot membrane with phospho-VASP (Ser157) antibody was analysed by G: BOX Chemi. The PGI_2 was a positive control and thrombin was a negative control. Lane 1, platelets were stimulated with thrombin (0.05 U/mL). Lane 2, platelets incubated with VIP (15 μM) and PSMα3 (30.7 μM) for 5 minutes. Lane 3, platelets were incubated with PSMα3 (30.7 μM) for 5 minutes; Lane 4, resting platelets. Lane 5, platelets were stimulated with PGI_2. PSMα3 induces VASP phosphorylation at residue Ser157 in

human platelets. Platelets were incubated with 30.7 µM of *S. aureus* PSMα3 for 5 minutes. (B) The quantitative analysis of the phosphorylation of VASP in Western immunoblot was made using ImageJ. The intensity of signal of Ser157-VASP was normalised to the loading control signal. Then ratio of each sample was calculated. These data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, **P≤0.01, ***P≤0.001).

VIP (6-28), VPAC1 antagonist, abrogated the action of *S. aureus* PSMα3. That means that *S. aureus* PSMα3 works on VPAC1 receptor (Figure 4.7). To conclude, this effect of *S. aureus* PSMα3 on platelets was meditated by VPAC1.

The effect of PSMα3 on platelet activity in platelets treated with VIP (6-28) (10 μM), VPAC1 receptor inhibitor, was studied to compare its impact with previous data (Figure 4.7). Platelets were pre-incubated with VIP (6-28) (10 μM) for 10 minutes then, *S. aureus* PSMα3 (15.38 and 30.7 μM) was added them and incubated for 5 minutes. Afterward, these platelets were stimulated with thrombin (0.05 U/mL) (Figure 4.7). Aggregation was measured for 5 minutes. PSMα3 losses its activity on platelet treated with the antagonist, VIP (6-28) (Figure 4.7A). The percentage aggregation was increased in platelets treated with VIP (6-28) cf platelets treated with PSMα3 (Figure 4.7B).

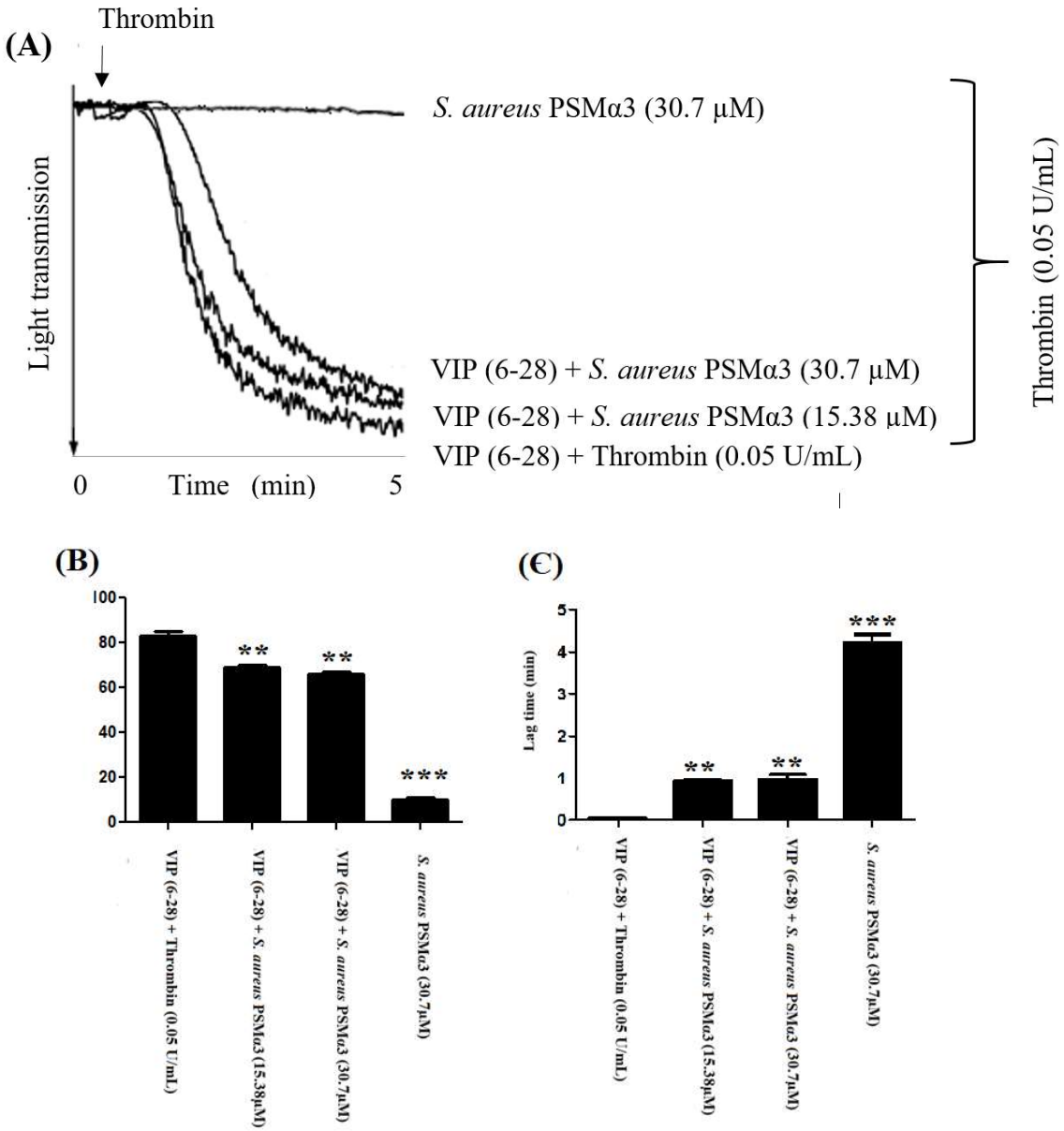


Figure 4.7: Effect of VPAC1 receptor antagonist on the action of *S. aureus* PSMα3. Representative traces. Washed human platelets ($4x10^8$ cells/mL) aggregation was recorded *via* a Chronolog 490-2d aggregometer for 5 minutes. **(A)** Platelets were pre-incubated with VIP (6-28) (10 µM) for 10 minutes then, *S. aureus* PSMα3 was added to them and incubated for 5 minutes. Afterwards the aggregation was recorded for 5 minutes. **(B)** Percentage of platelet aggregation. **(C)** The lag time of platelet aggregation. Platelets pre-incubated with VIP (6-28) showed loss of effect to *S. aureus* PSMα3 to cause inhibition of platelets. These data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, *P≤0.05, **P≤0.01, ***P≤0.001).

4.3 Conclusions

It has been reported that FPR2 has an ability to detect *S. aureus* PSMα3 in neutrophils (Kretschmer *et al.*, 2010), so experiments were conducted in order to investigate the role of FPR2 in human platelets and specifically its role in sensing PSMα3. Expression of FPR2 protein in human platelets was detected by Western immunoblot (Figure 4.1) and its localisation was determined by immunocytochemistry (Figure 4.2). MMK-1 was used to investigate functionality of FPR2. MMK-1 causes platelet aggregation (Figure 4.3). Activation assays showed that FPR2 was not involved in the interaction of PSMα3 and platelets (Figure 4.4).

Mass spectrometry analysis of the interaction between PSMα3 and platelets showed that VPAC1 receptor was involved in this interaction. Blocking of platelet VPAC1 abrogates the actions of *S. aureus* PSMα3 (Figure 4.6-4.7). Expression of VPAC1 receptor has been founds in megakaryocytes and platelets (Freson *et al.*, 2004). VPAC1 receptor is known to be a negative regulator of platelet function and causes inhibition of platelet activation (Freson *et al.*, 2004; Kis *et al.*, 1999). To conclude, *S. aureus* PSMα3 binding to platelets is mediated *via* VPAC1 receptor.

CHAPTER 5

Bioinformatics analysis of interaction between *S. aureus* PSMα3 and VPAC1 receptor

Chapter 5

5. Bioinformatics analysis of interaction between *S. aureus* PSMα3 and VPAC1 receptor

5.1 Introduction

Bioinformatics analysis is considered an invaluable tool for studying protein sequences and putative interactions between receptors and ligands. In this chapter, I-TASSER (Iterative Threading ASSEmbly Refinement), a bioinformatics tool, was used to predict secondary structure of *S. aureus* PSMα3. I-TASSER uses a threading technique that identifies structure templates from the Protein Data Bank (PDB). A VPAC1 receptor model was constructed by another tool called IntFOLD, which predicted the three-dimensional structure of VPAC1 by representing the correct folds of crystal structures in the PDB database using a template based method (McGuffin *et al.*, 2015; Roche *et al.*, 2011).

Physico-chemical properties of *S. aureus* PSMα3 and VPAC1 receptor were deduced from their amino sequences and calculated by ProtParam (http://www.expasy.org/tools/protparam.html) (Wilkins *et al.*, 1999). This tool provides various properties of *S. aureus* PSMα3 and VPAC1 such as theoretical isoelectric point (pI), total numbers of positively and negatively charged residues (Wilkins *et al.*, 1999).

Therefore, the objectives of this chapter were to provide a comprehensive *in silico* generated picture of how *S. aureus* PSMα3 may interact with platelet VPAC1 receptor through prediction of their structures. Hence, this computational approach would assist

achieving the goal of modelling *S. aureus* PSMα3-platelet VPAC1 receptor and also providing an understanding of this interaction.

5.2 Results

5.2.1 Prediction of secondary structure and possible binding sites of *S. aureus* PSMα3

The PDB database was searched for the three-dimensional structure of PSMα3 and the accession number 5KGY was found. The 5KGY structure was elucidated by nuclear magnetic resonance spectroscopy (NMR), and showed that PSMα3 has a high degree of amphiphilicity, with a helical shape (Towle *et al*., 2016). However, the model does not contain an N-formyl group, unlike natural PSMα3 secreted by *S. aureus* (Wang *et al*., 2007). N-formyl groups are important for the interaction of PSMα3 with host receptors (Kretschmer *et al.*, 2010). Therefore, a new model of PSMα3 containing an N-formyl group was generated.

The amino acid sequence of *S. aureus* PSMα3 (Table 2.18-A) was submitted to I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) to predict the secondary structure and possible binding sites of *S. aureus* PSMα3, as described in section 2.20.1. The result showed that amino acid residues; Met^{2}, Glu^{3}, Phe^{4} and Val^{5}, Ala^{6}, Lys^{7} and Leu^{8}, Phe^{9}, Lys^{10}, Phe^{11}, Phe^{12} and Lys^{13}, Asp^{14}, Leu^{15}, Leu^{16} and Gly^{17}, Lys^{18}, Phe^{19} and Leu^{20} provided a high degree of α-helicity in the putative secondary structure of *S. aureus* PSMα3 (Figure 5.1) and also putative coils were observed at amino acid residues Phe^{1} and Asn^{23} in the secondary structure of *S. aureus* PSMα3. Moreover, ligand-binding sites on the *S. aureus* PSMα3 were predicted at amino acid residues; Val^{5}, Ala^{6} and Lys^{7}, Leu^{8},

Phe[9] and Lys[10], Phe[11], Phe[12] and Lys[13] Asp[14], Leu[15] and Leu[16],Gly[17] and Leu[20] (Figure 5.2).

Furthermore, the ExPASy-ProtParam tool (http://web.expasy.org/protparam) (section 2.20.2), was used to identify the relevant physiochemical properties of *S. aureus* PSMα3. The theoretical pI of *S. aureus* PSMα3 was 9.53, meaning that *S. aureus* PSMα3 is a highly basic peptide. The total number of positively charged residues (Arg + Lys) is 4, while the total number of negatively charged residues (Asp + Glu) is 2. The most frequent amino acids were determined: Phe with 26.1%, Leu with 17.4% and Lys with 17.4% composition.

(A)

(B)

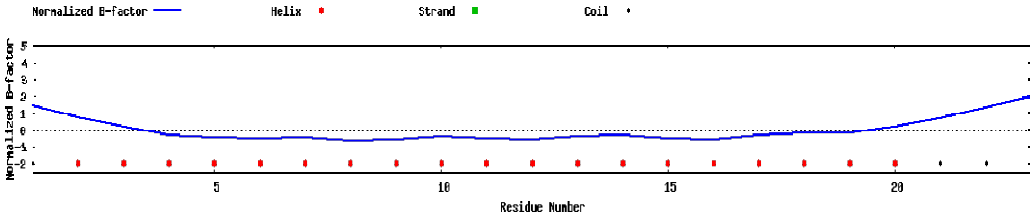


Figure 5.1: Model of the *S. aureus* PSMα3. (A) Diagram of putative secondary structure of *S. aureus* PSMα3, predicted by I-TASSER. Met^{2} to Leu^{20} amino acid residues that appear in red colour form the rod-like structure (α-helices). This α-helicity is tightly coiled by Phe^{1} and Asn^{23} that appear in green. **(B)** Normalised B-factor showing the quality of the predicted model of *S. aureus* PSMα3.The C-score = 0.00, TM-score = 0.71 ±0.11 and 1.3 ±1.3Å.

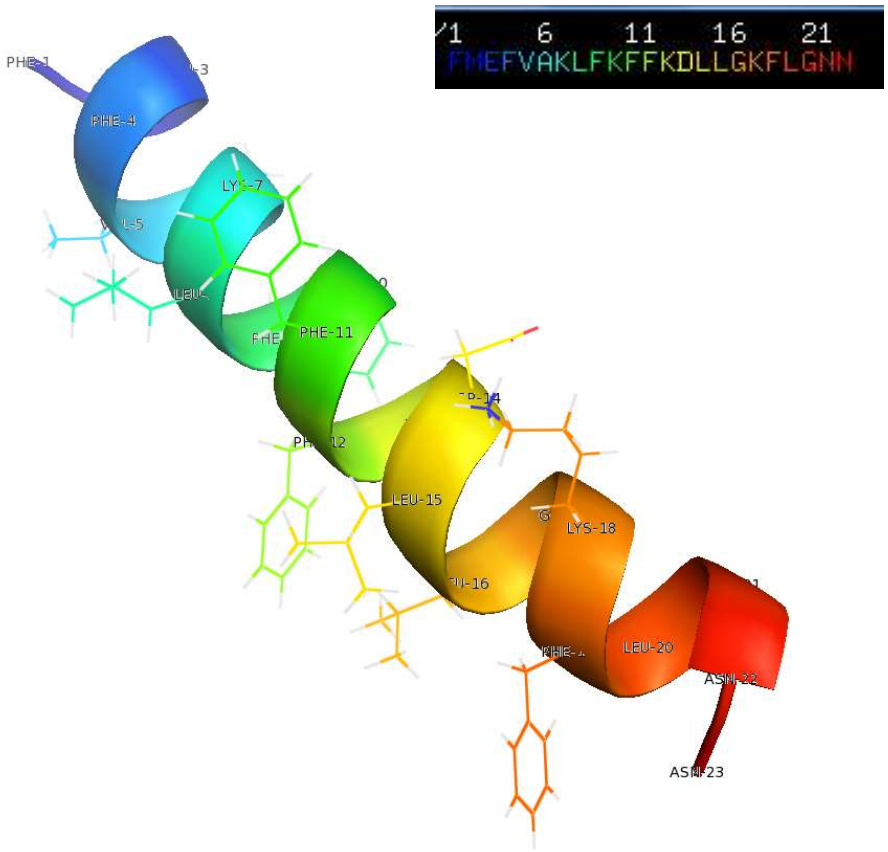


Figure 5.2: Ligand-binding sites on model of the *S. aureus* PSMα3. Diagram of putative secondary structure of *S. aureus* PSMα3 combine with ligand-binding sites that appears as stick structure. Fourteen residues were predicted as ligand-binding sites by I-TASSER. The C-score= 0.23.

5.2.2 Modelling of VPAC1 receptor

The PDB database was interrogated for a three-dimensional structure of VPAC1 receptor. Several models of VPAC1 were found in this database, which were obtained by NMR or X-ray crystallography and appear as complexes with different peptides. However, those models lack the whole N-terminal extracellular domain of the VPAC1 receptor. The N-terminal extracellular domain of this receptor plays a dominant role in recognition of its ligands (Couvineau *et al*., 2006). Thus, a model of VPAC1 receptor was constructed by IntFOLD Server.

In this work, the amino acid sequence of VPAC1 receptor (Table 2.18-B) was submitted to IntFOLD (Version 3.0) and the top-scored model was downloaded in PDB format from the IntFOLD Server. Following submission, 104 models were predicted and almost all of them were based on either activated glucagon-like peptide-1 receptor (PDB 5vai) (Figure 5.4B), the structure of the human glucagon class b receptor (PDB 4l6r) (Figure 5.4C) or both.

These models were ranked according to their global model quality score. The top 5 models of VPAC1 were presented in the Figure 5.3, model 1 (Figure 5.3A), model 2 (Figure 5.3B), model 3 (Figure 5.3C), model 4 (Figure 5.3D) and model 5 (Figure 5.3E). These models are arranged from the high to low score of global quality. The global quality scores of these top 5 models are greater than 0.4 which indicates more complete and confident models.

Template modeling score (TM) was used to measure the similarity between the structures of PDB 5vai and PDB 4l6r with the native structure of VPAC1. The TM-score of PDB 5vai is 0.58859 and PDB 4l6r is 0.59988. Based on these TM-scores, structures of PDB 5vai and PDB 4l6r share the same fold with the native structure of VPAC1.

The top score of the predicted model of VPAC1 receptor structure was based on both templates of PDB 5vai and PDB 4l6r with high reliability (Figure 5.4- 5.5). The judgement of the high reliability of this model was based on the score of modeling-confidence. The overall global quality score of this predicted model of VPAC1 receptor was 0.4611 with a P-value of 7.475E-3. This score indicated that this prediction is a more complete and confident model, which is very similar to the native structure of VPAC1. The high reliability of this model was also shown by the excellent β-factor. This β-factor was represented by colour intensity (Figure 5.4A).

Physiochemical properties of VPAC1 receptor were determined by the ExPASy-ProtParam tool (http://web.expasy.org/protparam). The result showed that the theoretical pI of the VPAC1 receptor was 8.52, which indicated it to be a basic protein. The total number of positively charged residues (Arg + Lys) is 40, while the total number of negatively charged residues (Asp + Glu) is 34. Leu, which makes up 11.6% of amino acid residues was the most frequent amino acid in the VPAC1 receptor.

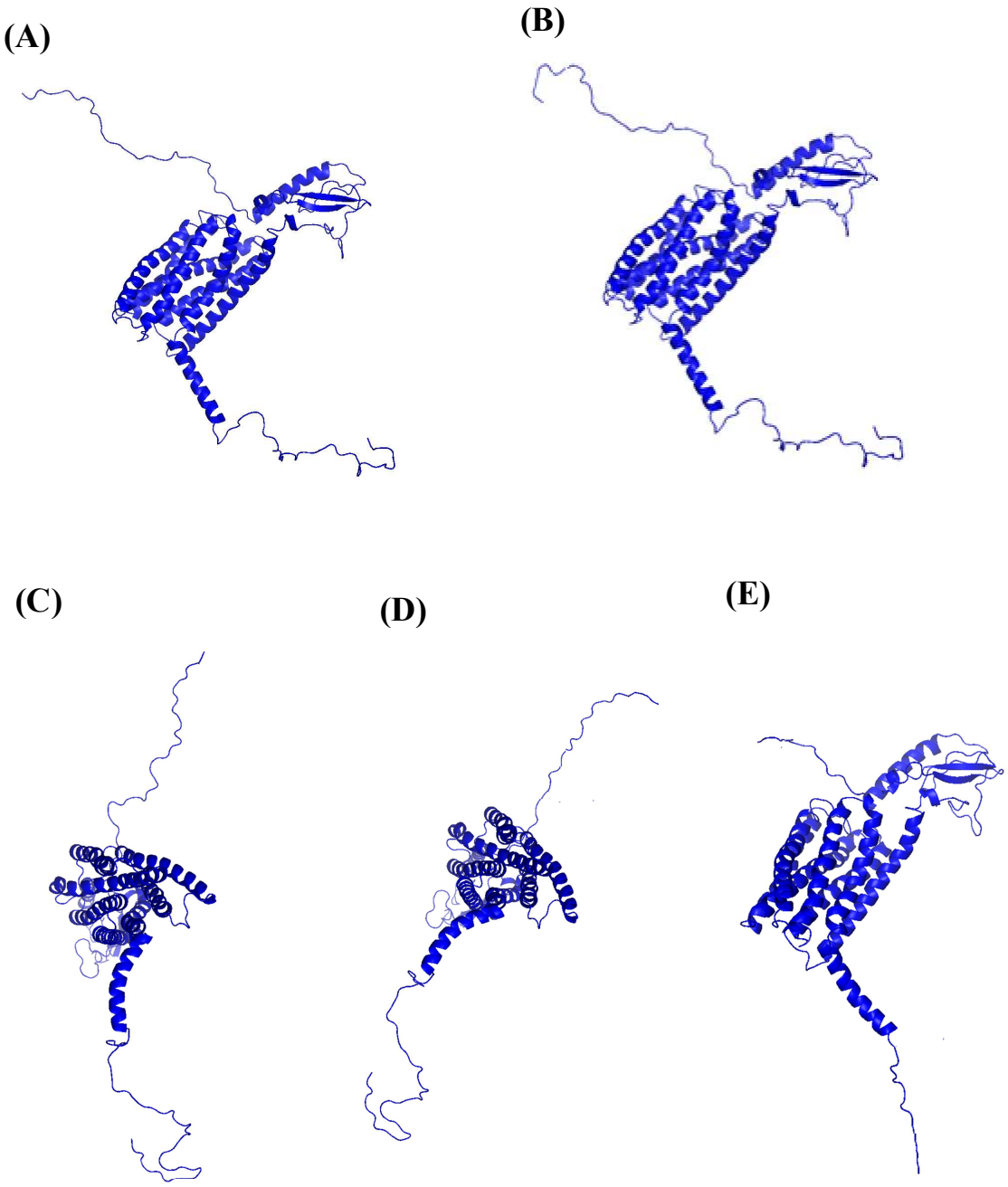


Figure 5.3: Top 5 models of VPAC1. Ribbon diagram of top 5 models of VPAC 1 were predicted by IntFOLD3.0; model 1**(A)**, model 2 **(B)**, model 3 **(C)**, model 4 **(D)** and model 5 **(E)**. The overall global quality scores of these predicted models of VPAC1 receptor are 0.4611, 0.4507, 0.4366, 0.4351 and 0.4332 for model 1, model 2, model 3, model 4 and model 5, respectively.

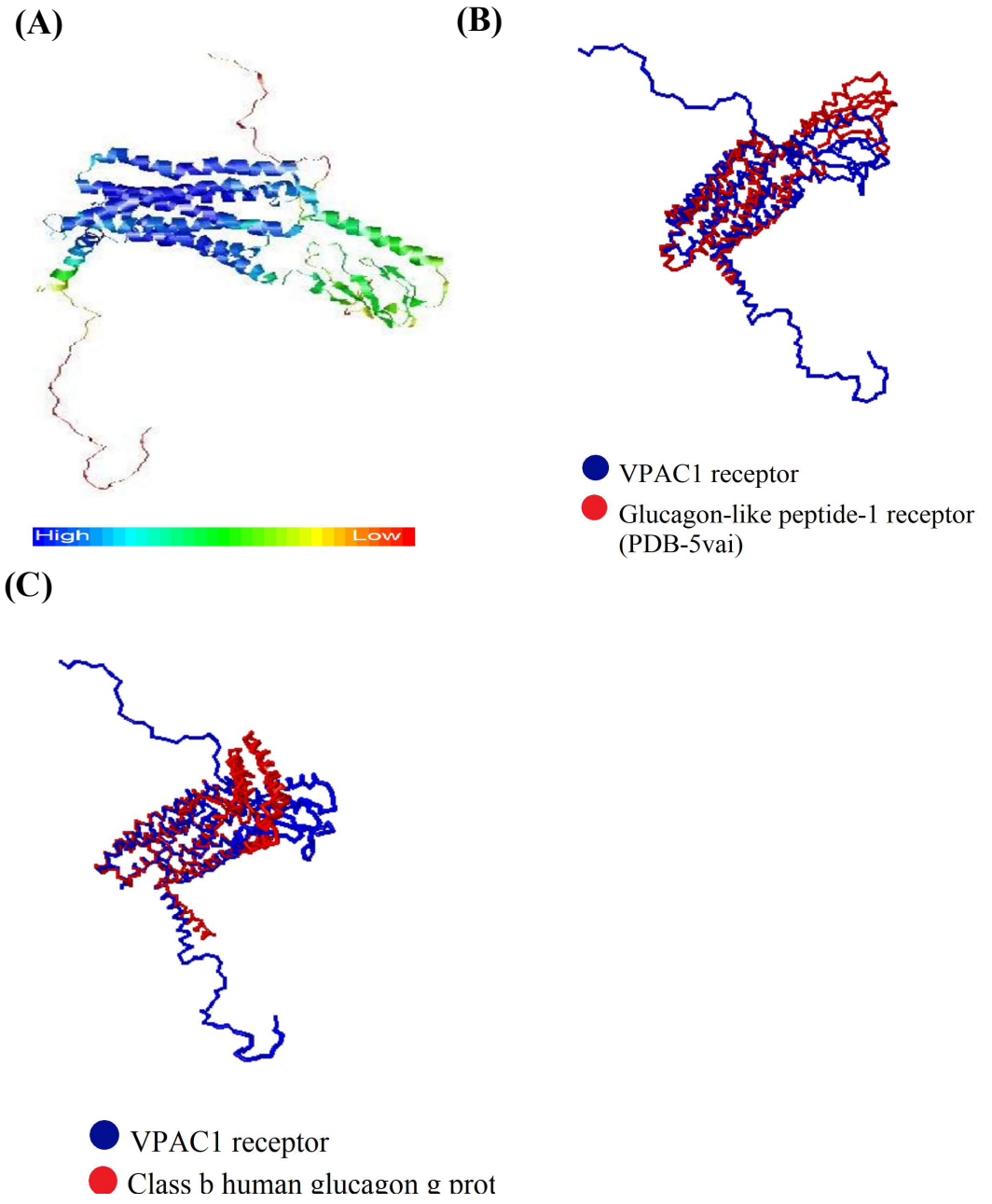


Figure 5.4: Model of the VPAC1 receptor. (A) Ribbon diagram of VPAC1 receptor structure, predicted by IntFOLD3.0, and β-factor colour intensity displayed as reverse rainbow scheme that shows the quality of prediction. High accuracy- blue, low accuracy- green to red. **(B)** VPAC1 receptor interaction with glucagon-like peptide-1 receptor (PDB 5vai), RMSD:3.7, TM-score: 0.58859 or **(C)** with class b human glucagon g protein coupled receptor (PDB 4l6r), RMSD: 3.47,TM-score: 0.59988.

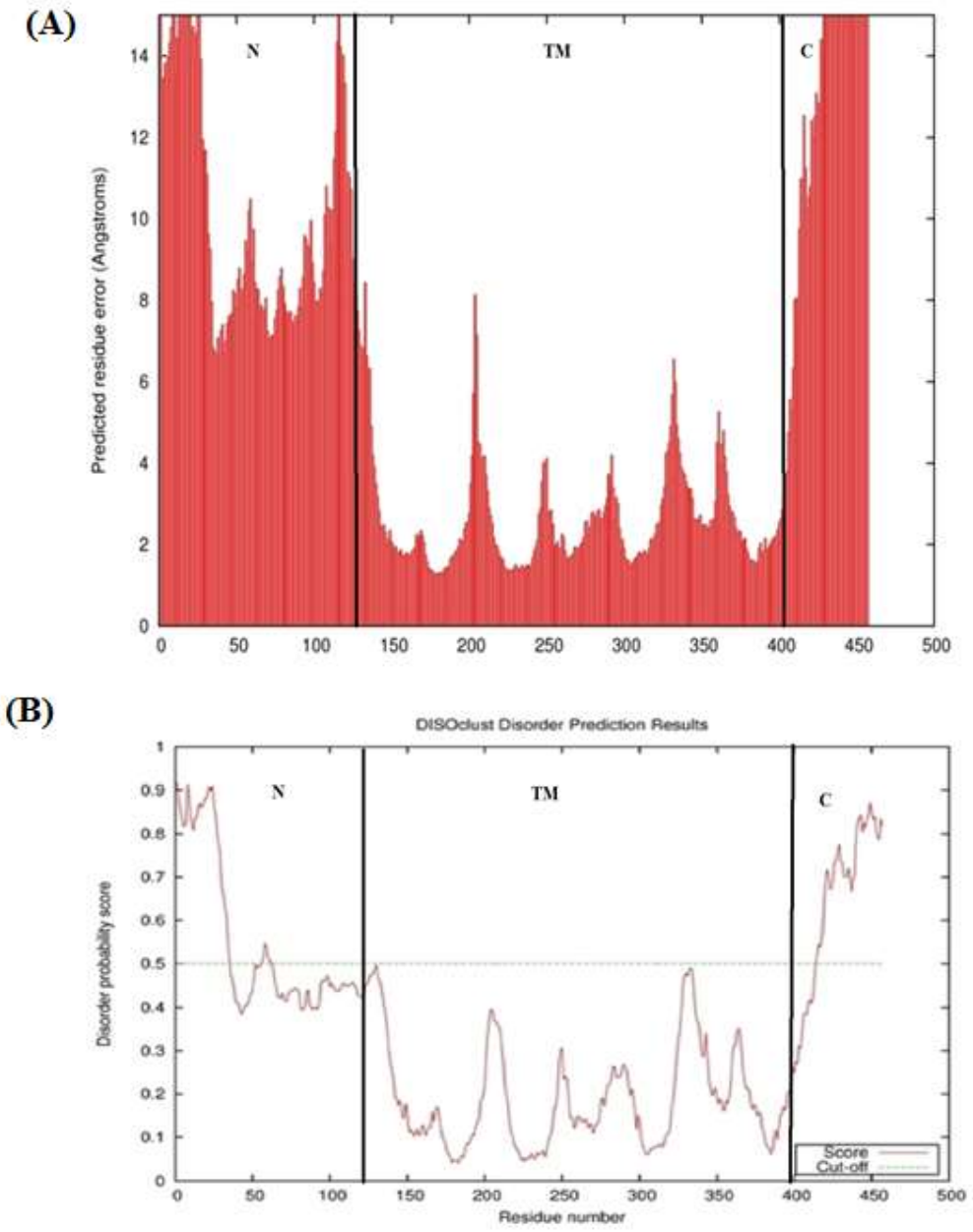


Figure 5.5: Per-residue error and disorder prediction of model of VPAC1 receptor. **(A)** Per-residue error in A which is the highest scoring model of VPAC1 receptor based on Glucagon-like peptide-1 receptor (PDB 5vai). **(B)** Disorder prediction of the highest scored model of VPAC1 receptor based on Glucagon-like peptide-1 receptor (PDB 5vai). The horizontal green dotted line shows that cut-off value (0.5) which is a good quality prediction. The vertical black dotted line in both **A** and **B** showed the division between N-terminus and transmembrane domains (TM) and C- terminus of VPAC1 receptor.

Prediction of 3D structures of proteins can have different approaches: homology modelling, threading (fold recognition) or by ab-initio methods (Deng *et al.*, 2018). In this regard, the model of VPAC1 (Figure 5.4) was compared to the published models (Tan *et al*., 2006; Couvineau *et al*., 2006). Tan *et al*. used homology modelling to generate 3D model of VPAC1. Sequence alignment was done between mCRF2 receptor and VPAC1. Sequence identity which represents alignment accuracy, was 22%; (Tan *et al*., 2006) that falls into the "twilight" zone (<25-30%) (Rost, 1999). The fold recognition approach is essential for identifying more accurately, 3D modelling for a target (Godzik, 2003; McGuffin, 2008). In this regard, the fold recognition method was used to generate a high accuracy 3D structure for VPAC1 (Figure 5.4). This method is necessary to find the correct fold of a target protein sequence (Godzik, 2003; McGuffin, 2008). The IntFOLD Server uses a combination of fold recognition and homology modelling in the construction of models (Figure 5.3 and 5.4). In sharp contrast to template selection by Tan *et al*., they used a different template: mCRF2 receptor (Tan *et al*., 2006). Couvineau *et al.* (2006) used rhodopsin-like receptor, which is a class A GPCR, as a template for construction of the model of VPAC1 (Couvineau *et al*., 2006). While in this work, glucagon-like peptide-1 receptor and class b human glucagon were used as the template for VPAC1 (Figure 5.3 and 5.4) since, class A GPCRs have low overall sequence identity with class B GPCRs, including VPAC1 (Pierce *et al.*, 2002). Based on these differences in these published models, this could affect the docking partner in terms of selection of binding sites or residues that could have an impact on hydrophobic or hydrophilic interactions. This might affect the underlying molecular interaction involved.

5.2.3 Identification of key amino acid residues of VPAC1 receptor that involved in interaction with *S. aureus* PSMα3

Further work was carried out to identify binding sites of *S. aureus* PSMα3 on VPAC1. For this purpose, MEGADOCK was used to dock *S. aureus* PSMα3 within VPAC1. ProQdock was used to check the quality of the docking model.

Potential binding sites for *S. aureus* PSMα3 on VPAC1 receptor were visualised by MacPyMOL1.3. Based on this model of *S. aureus* PSMα3 and VPAC1 receptor complex, His^{47} and Leu^{51} residues in N-terminal domain of VPAC1 receptor were identified as possible binding sites on VPAC1 receptor for *S. aureus* PSMα3 (Figure 5.6-5.7).

Phe^{1}, Met^{2} and Phe^{4} on the *S. aureus* PSMα3 were predicted as essential for the interaction with VPAC1 (Figure 5.7). These residues in the binding model provides information about how this inhibitory effect of the *S. aureus* PSMα3 on platelets may occurred.

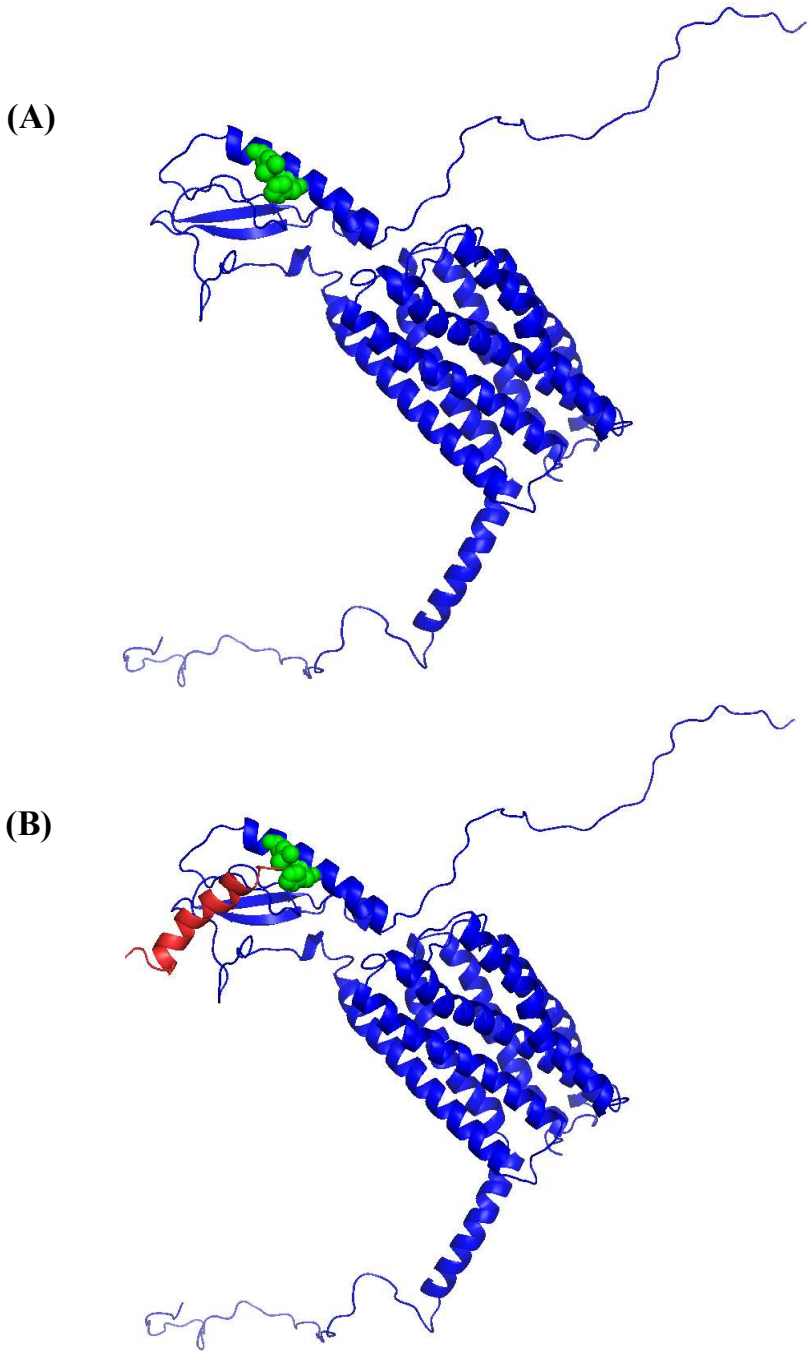


Figure 5.6: Docking model of interaction of PSMα3 with VPAC1 receptor. Model of interaction of *S. aureus* PSMα3 with VPAC1 receptor. (A) VPAC1 receptor without *S. aureus* PSMα3. (B). VPAC1 receptor with *S. aureus* PSMα3. Binding sites residues of VPAC1 for *S. aureus* PSMα3 on VPAC1 receptor were presented by green colour. Megadock 4.0 software was used for generating of this model of PSMα3-VPAC1 receptor interaction and it was validated by ProQdock. The score is 0.92545, which indicated that this docking model has a high quality.

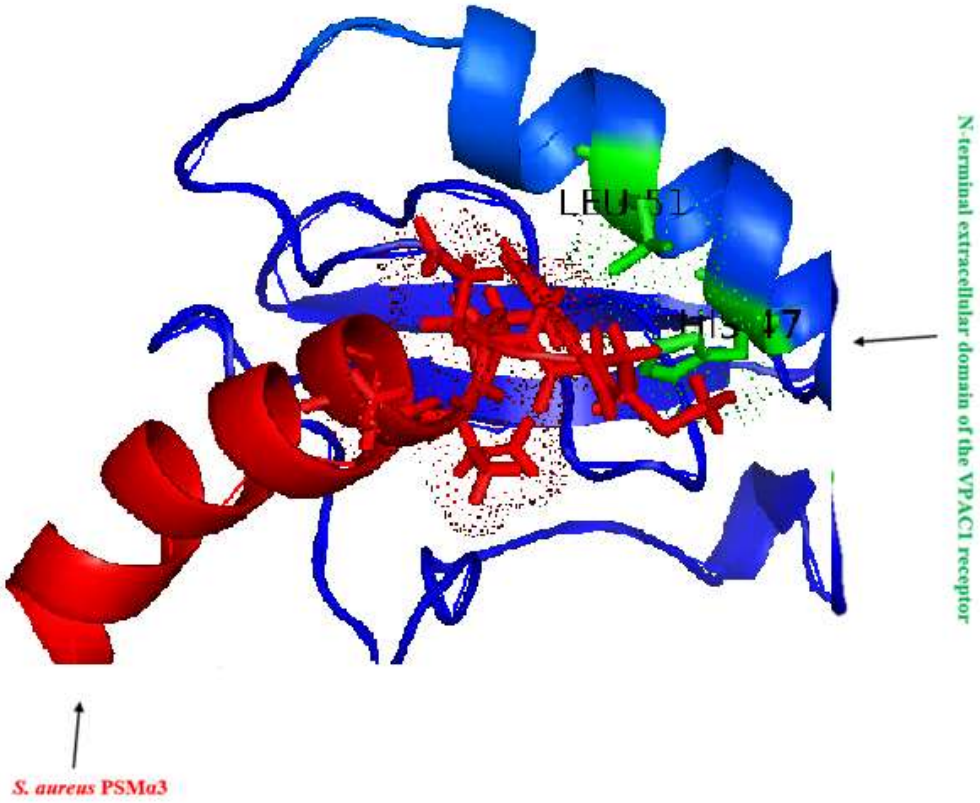


Figure 5.7: Essential residues of VPAC1 receptor. Residues (coloured in green) in the N-terminal domain of the receptor that were required for the interaction of the *S. aureus* PSMα3 (coloured in red). His^{47} and Leu^{51} in the N-terminal domain of VPAC1 receptor were found to be possible binding site for PSMα3. A contact between His^{47} of VPAC1 and Phe^{1} and Met^{2} residue in PSMα3. In addition, there is a contact between Leu^{51} of VPAC1 with Phe^{4} of PSMα3. MacPyMOL1.3 was used for visualisation of this model.

This docking model of the interaction of PSMα3 with VPAC1 was compared to docking models of the interaction of VIP and VPAC1. Tan *et al.* (Tan *et al.*, 2006), used the HADDOCK 1.2 program (Dominguez *et al.*, 2003) to dock VIP into VPAC1 (Tan *et al.*, 2006) *in silico*. MEGADOCK 4.0 was used to generate the docking model of the interaction of PSMα3 with VPAC1. Comparing these programs, MEGADOCK 4.0 is superior to HADDOCK. MEGADOCK 4.0 creates a docking model in a grid-based 3D space, using a fast Fourier transform (FFT) (Ohue *et al.*, 2014). However, the validity of these docking models of PSMα3-VPAC1 (Figure 5.6) and VIP-VPAC1 were remarkably good quality.

Furthermore, several docking models were generated by MEGADOCK. ProQdock was then used to rank these models according to their quality score and only the top-scored model was used in this work (Figure 5.6 and 5.7). Based on docking results, the top 5 models on ProQdock. They have the same binding sites are His^{47} and Leu^{51} in the N-terminal domain of VPAC1 (Figure 5.7). In addition, there are other binding sites for PSMα3, which are Trp^{73}, Pro^{74} and Val^{81}-Ile^{84}. This variation in binding sites among these models could be due to template selection or underlying mechanisms (algorithms) of the docking software (Pagadala *et al.*, 2002). Thus depending on docking results, PSMα3 may not bind at all to the same residues as VIP. For instance, PSMα3 binds to His^{47} and Leu^{51}, Trp^{73}, Pro^{74} and Val^{81}-Ile^{84}. While VIP binds Asp^{107}, Gly^{116} and Cys^{122} in the N-terminal domain of VPAC1.

S. aureus PSMα3 and VIP share some common properties; (1) they are short peptides with 23 and 28 amino acid residues, respectively (Laabei *et al.*, 2014; Nicole *et al.*, 2000). (2) PSMα3 and VIP exhibit an obvious tendency to form α-helices. Both of them have a

central α-helix with tightly coiled N- and C terminus (Nicole *et al*., 2000). Diagram of putative secondary structure of *S. aureus* PSMα3 showed that α-helices are formed between Met^{2} and Leu^{20} residues (Figure 5.1). This α-helicity is tightly coiled by Phe^{1} and Asn^{23} (Figure 5.1). (3) N-terminal domain of VPAC1 is the binding site for both PSMα3 and VIP (Couvineau *et al.*, 2012). Data reveal that Phe^{1}, Met^{2} and Phe^{4} residues of PSMα3 interact with VPAC1 (Figure 5.7). These residues directly interact with His^{47} and Leu^{51} residues of N-terminal domain of VPAC1 (Figure 5.7). VIP interacts with N-terminal domain of VPAC1 (Couvineau *et al.*, 2006). Asp^{107}, Gly^{116}, and Cys^{122} in the N-terminal domain of VPAC1 are crucial for VIP binding (Couvineau *et al.,* 2006). Moreover, these residues in the N-terminal domain are involved in the activation of this receptor (Tan *et al.*, 2006).

PSMα3 and VIP have similar in physiological effects on platelets. VIP inhibts platelet aggregation (Cox *et al.*, 1984), by causing an increase levels of cAMP and activation of PKA within platelets (Cox *et al.,* 1984; Kishi *et al.*, 2000). VIP achieves these effects by direct interaction with VPAC1 (Kishi *et al.,* 2000). This work demonstrated that PSMα3 has similar effects on platelet activity through inhibition of platelet activation and aggregation. PSMα3 interacts with platelets via VPAC1. Based on reviews the present knowledge regarding VIP-VPAC1 interaction and docking model (Figure 5.6-5.7). These data predict that the initial contact between PSMα3 and VPAC1 on platelets, is via a specific interaction. These observations were in good agreement with natural ligands of VPAC1 e.g. VIP (Laburthe *et al.*, 2002). Finally, according to docking results, PSMα3 and VIP may bind to different residues on VPAC1. This could be due to the methods that were used to generate the models and this could affect binding sites that are involved by the molecules (Pagadala *et al.*, 2002).

5.3 Conclusions

The structural bioinformatics analyses and results have indicated the potential binding specificity of *S. aureus* PSMα3 and VPAC1 receptor. Based on the models in Figure 5.7, the receptor VPAC1 shows a likely strong binding affinity with PSMα3 which is in accordance with the results obtained using mass spectrometry.

The residues; His^{47} and Leu^{51} in N-terminal domain of VPAC1 receptor were predicted essential for the interaction with PSMα3.

PSMα3 has Phe^{1}, Met^{2} and Phe^{4} that were predicted essential for the interaction with VPAC1. These data predict how the initial interaction between PSMα3 and VPAC1 on platelets may occur.

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CHAPTER 6

S. aureus PSMα3 modulates human platelet functions *via* activation of cAMP-dependent PKA

Chapter 6

6. *S. aureus* PSMα3 modulates human platelet functions *via* activation of cAMP-dependent PKA

6.1 Introduction

The response of platelets to their external environment is dependent on the balance of different signals; different signalling pathways can be divided into activatory and inhibitory. Activatory signals released from the injured vasculature, or an invading pathogen, can initiate platelet activation. On other hand, inhibitory signals secreted from healthy endothelial cells can suppress activation of platelets (Mitchell *et al.*, 2008). Modulation of these signalling pathways of platelets by invaded pathogens could be beneficial for them when establishing infection (Athanasopoulos *et al.*, 2006; Shannon *et al.*, 2005).

The intracellular signalling in human platelets, treated with *S. aureus* PSMα3, was investigated in this chapter, as previous results demonstrated that *S. aureus* PSMα3 was inhibitory for platelet activation and aggregation. More specifically, the aim of this chapter was to determine the functional inhibitory pathway(s) of *S. aureus* PSMα3 and to identify the potential mechanism(s).

6.2 Results

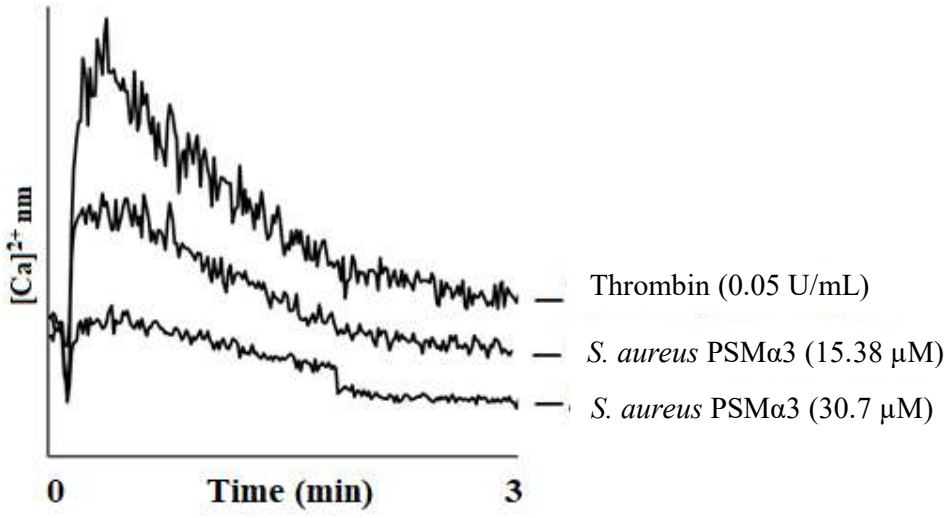
6.2.1 Effect of *S. aureus* PSMα3 on platelet intracellular calcium

The effect of *S. aureus* PSMα3 on calcium mobilisation following thrombin and CRP-XL induced platelet activation was studied. PRP was incubated with Fura-2AM dye and

loaded platelets were incubated with *S. aureus* PSMα3 for 5 minutes prior to activation with thrombin and their fluorescence was recorded for 3 minutes by spectrofluorimetry.

Data showed that *S. aureus* PSMα3 inhibits Ca^{2+} flux from platelets, in a dose-dependent manner, which prevents platelet activation in response to 0.05 U/mL of thrombin (Figure 6.1A) or 0.5 µg/mL of CRP-XL (Figure 6.2A). *S. aureus* PSMα3 (30.7 µM) causes 65% of inhibition and 15.38 µM of *S. aureus* PSMα3 causes 40.17% of inhibition (Figure 6.1B). While 67% inhibition was caused by 30.7 µM of *S. aureus* PSMα3 and 34% inhibition was caused by 15.38 µM of *S. aureus* PSMα3 in response to CRP-XL (Figure 6.2B).

(A) Thrombin



(B) Percentage of calcium mobilisation

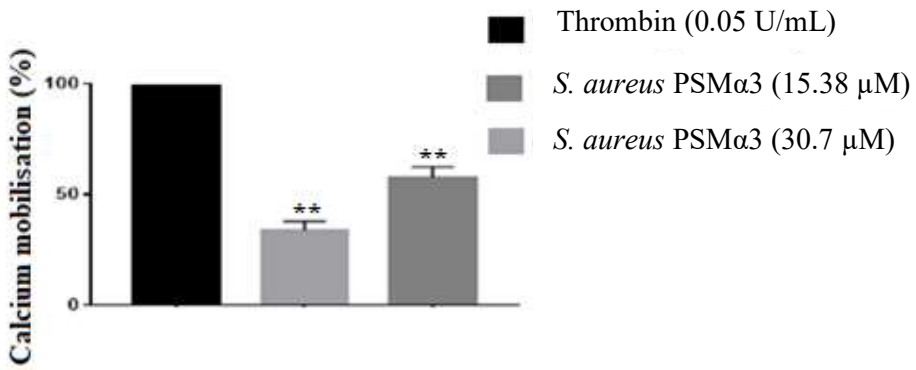
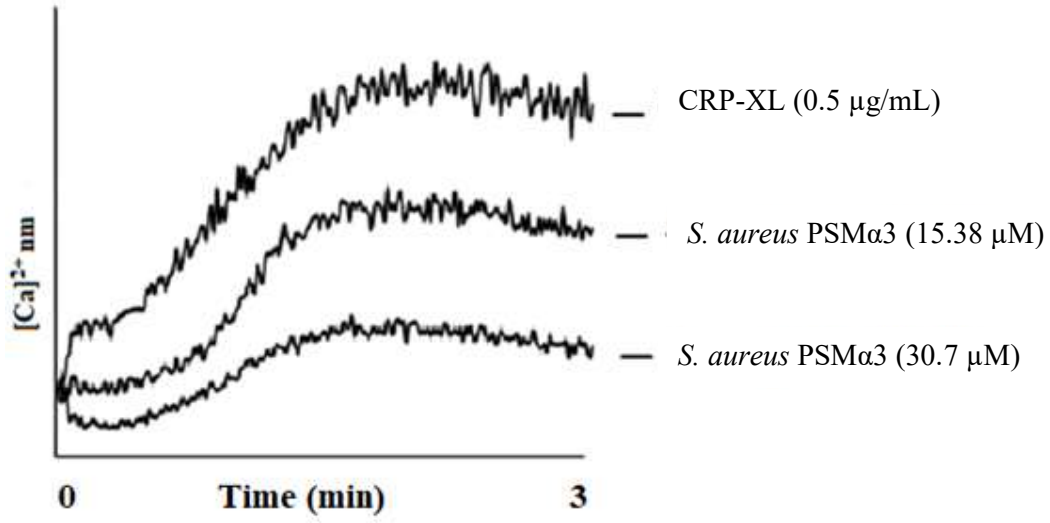


Figure 6.1: *S. aureus* PSMα3 inhibits calcium mobilisation in response to thrombin. **(A)** A representative trace. Fura-2AM-loaded platelets were pre-incubated with *S. aureus* PSMα3 (15.38 and 30.7 μM) for 5 minutes. Afterwards platelets were activated by thrombin (0.05 U/mL) and were recorded for 3 minutes by spectrofluorimetry. The *S. aureus* PSMα3 showed ability to inhibit calcium mobilisation in platelets. **(B)** Percentage inhibition at peak of cytoplasmic calcium concentration compared with thrombin. These data represent ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test,**P≤0.01).

(A) CRP-XL



(B) Percentage of calcium mobilisation

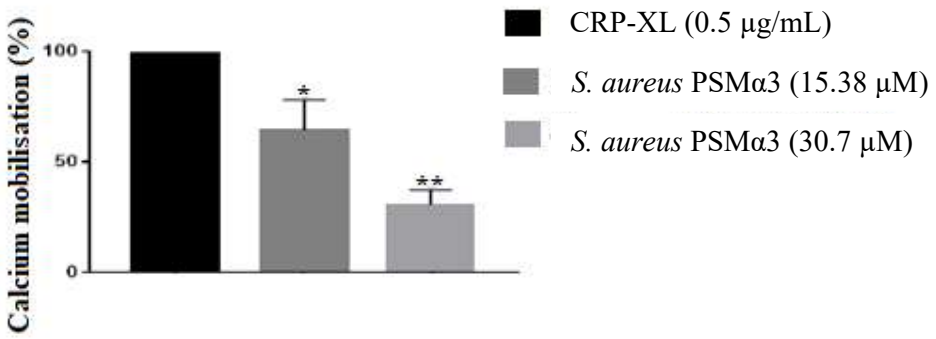


Figure 6.2: *S. aureus* PSMα3 inhibits calcium mobilisation in response to CRP-XL. **(A)** A representative trace. Fura-2AM-loaded platelets were pre-incubated with *S. aureus* PSMα3 (15.38 and 30.7 µM) for 5 minutes. Afterwards platelets were activated by CRP-XL (0.5 µg/mL) and were recorded for 3 minutes by spectrofluorimetry. The *S. aureus* PSMα3 showed ability to inhibit calcium mobilisation in platelets. **(B)** Percentage inhibition of peak cytoplasmic calcium concentration compared with CRP-XL (n=3; one-way ANOVA with Tukey as a post hoc test,*P≤0.05, **P≤0.01).

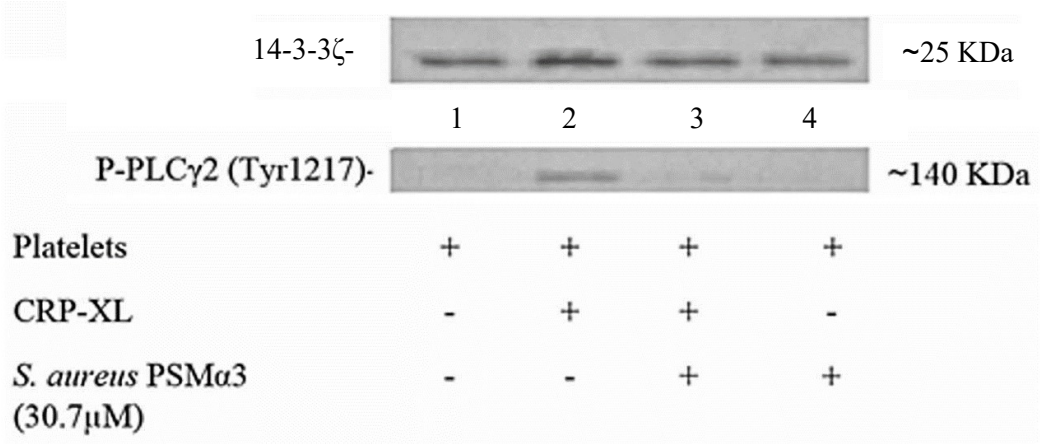
6.2.2 Effect of *S. aureus* PSMα3 on platelet calcium signalling

According to the previous data, *S. aureus* PSMα3 is able to inhibit Ca^{2+} mobilisation (Figure 6.1-6.2), fibrinogen binding and degranulation of platelets (Figure 3.7-3.9). This experiment was designed to explain why there was a reduction in the level of the Ca^{2+} release. In this experiment, signalling events and their effect upstream and downstream of Ca^{2+} flux were examined.

6.2.2.1 The effect of *S. aureus* PSMα3 on upstream of Ca^{2+} signalling

This experiment was designed to study the role of *S. aureus* PSMα3 on Ca^{2+} upstream signalling by analysing its effect on activation of PLC. Western immunoblot analysis was used for detection of activity of PLCγ2 in human platelets treated with *S. aureus* PSMα3. The incubation time of platelets and PSMα3 was 5 minutes, and then they were stimulated with 0.5 µg/mL of CRP-XL for 90 seconds. Resting platelets were used as a negative control and platelets stimulated with the same dose of CRP-XL were used as a positive control. These data show that PSMα3 did not cause phosphorylation of PLCγ2 in platelets (Figure 6.3, Lane 3 and Lane 4), when compared to the positive control (Figure 6.3, Lane 2). Indeed, *S. aureus* PSMα3 inhibited PLCγ2 phosphorylation by a physiological agonist. This observation correlates with the pervious finding of inhibition of calcium mobilisation. Inhibition of PLC leads to impaired production of IP3, that leads to a reduction in Ca^{2+} flux.

(A)



(B)

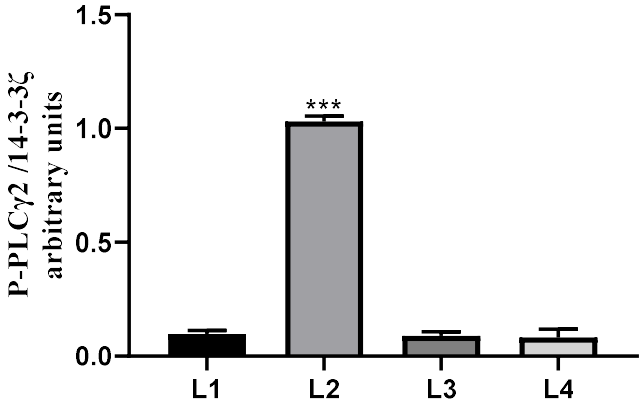


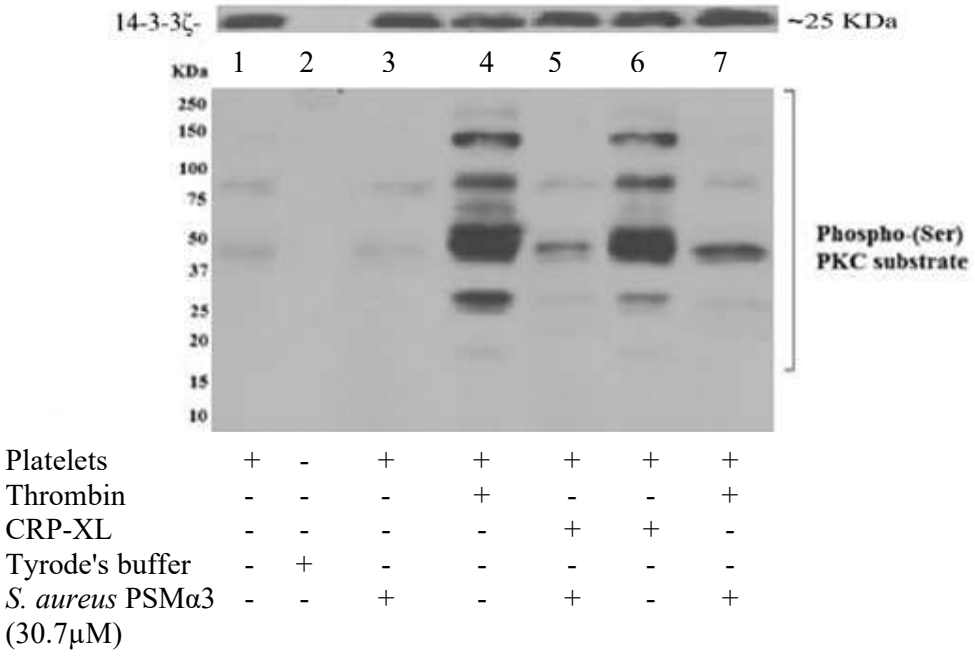
Figure 6.3: Inhibition of PLCγ2 phosphorylation in human platelets treated with *S. aureus* PSMα3. Platelets were incubated with 30.7 μM of *S. aureus* PSMα3 for 5 minutes. CRP-XL was a positive control and resting platelets were a negative control. **(A)** Western blot with phospho-PLCγ2 (Tyr1217) antibody, analysed by G: BOX Chemi (n=3). Lane 1, resting platelets; Lane 2, platelets treated platelets were incubated with CRP-XL (0.5 μg/mL); Lane 3, platelets were incubated with PSMα3 (30.7μM) for 5 minutes and then stimulated with 0.5 μg/mL of CRP-XL; and Lane 4, platelets were stimulated with CRP-XL (0.5 μg/mL). **(B)** The quantitative analysis of the phosphorylation of PLCγ2 in western immuno-blot was made by using ImageJ program. The intensity signal of phosphorylation of PLCγ2 was normalised to the loading controls signal. Then the ratio of each sample was calculated. These data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, ***P≤0.001).

6.2.2.2 The effect of *S. aureus* PSMα3 on downstream of Ca^{2+} signalling

PKC is a member of the protein kinase family and its function is to regulate other proteins by phosphorylation of their hydroxyl groups on serine and threonine residues (Mellor *et al.*, 1998). In platelets, PKC becomes activated by elevated Ca^{2+} or DAG concentrations (Mellor *et al.*, 1998). PKC is divided into three isoforms which are PKCβI/II, PKCγ and PKCα that control platelets *via* phosphorylation of serine and threonine (Mellor *et al.*, 1998).

The effect of *S. aureus* PSMα3 on PKC activation was examined. Total phospho-(Ser) PKC substrate antibody from cell signalling was used to detect activation of PKC. This detection was carried out with washed human platelets treated with PSMα3 using Western immunoblot. Thrombin and CRP-XL were used as positive controls, while untreated resting platelets were used as a negative control. This experiment was carried with 30.7 μM of PSMα3 and human platelets. The finding showed inhibition of PKC in platelets treated with *S. aureus* PSMα3 (Figure 6.4; Lane 3, Lane 5 and Lane 7). This inhibition correlates with inhibition of degranulation and fibrinogen binding of platelets due to inhibition downstream of Ca^{2+}.

(A)



Platelets	+	-	+	+	+	+	+
Thrombin	-	-	-	+	-	-	+
CRP-XL	-	-	-	-	+	+	-
Tyrode's buffer	-	+	-	-	-	-	-
S. aureus PSMα3 (30.7μM)	-	-	+	-	+	-	+

(B)

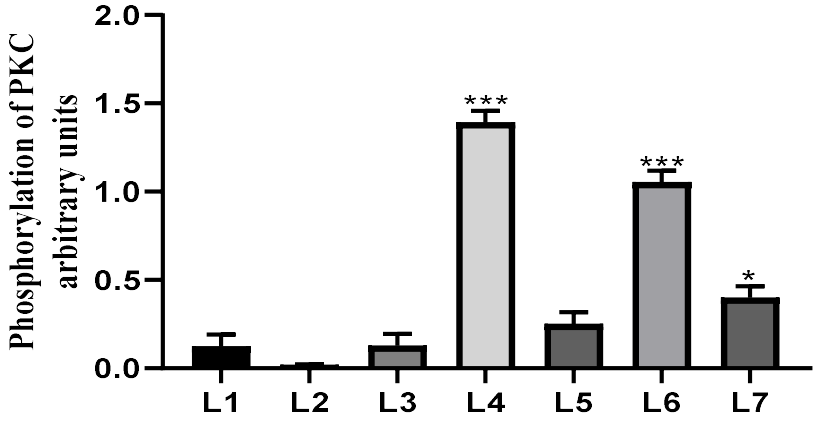


Figure 6.4: Effect of *S. aureus* PSMα3 on PKC activation. (A) Human platelets were treated with *S. aureus* PSMα3 (30.7 μM) and incubated for 5 minutes. After that, these treated platelets were incubated with thrombin or CRP-XL. Total phospho-(Ser) PKC substrate antibody was used for detecting phosphorylation of PKC in these platelets. The membrane of the Western blot was examined by G: BOX Chemi (n=3). Lane 1, resting platelets; Lane 2, only tyrode's buffer; and Lane 3, platelets were incubated with PSMα3 (30.7 μM) for 5 minutes. Lane 4, platelets were stimulated with 0.05 U/mL of thrombin; and Lane 5, platelets were incubated with PSMα3 (30.7 μM) for 5 minutes and then stimulated with 0.5 μg/mL of CRP-XL. Lane 6, platelets were stimulated with 0.5 μg/mL of CRP-XL; and Lane 7, platelets incubated with PSMα3 (30.7μM) for 5 minutes and then stimulated with 0.05 U/mL of thrombin. Results showed that PSMα3 was able to inhibit phosphorylation of PKC by itself, Lane 3, and also in presence of platelet agonists,

Lane 5 and Lane 7, cf controls, Lane 4 and Lane 6. **(B)** The quantitative analysis of the phosphorylation of PKC in western immuno-blot was made using ImageJ. The intensity of signal of phosphorylated PKC was normalised to the loading control signal. Then the ratio of each sample was calculated. These data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, *P≤0.05, **P≤0.01, ***P≤0.001).

6.2.3 Effect of *S. aureus* PSMα3 on VASP phosphorylation

VASP proteins are found in a variety of human cells such as platelets, fibroblasts, endothelial cells and smooth muscle cells (Eigenthaler *et al.*, 1992; Halbrugge *et al.*, 1989). VASP plays roles in these cells *via* cell-cell contacts, by binding to vinculin protein, regulation of motility and cytoskeletal structure of cells by the activation of integrin and modulation of actin polymerisation (Massberg *et al.*, 1999). VASP is highly expressed in human platelets and has two main phosphorylation sites, Serine-239 and Serine-157. Ser239 is phosphorylated by PKG, while Ser157 is phosphorylated by PKA (Kwiatkowski *et al.*, 2003).

S. aureus PSMα3 showed an inhibitory effect on platelet activation and aggregation in response to thrombin and CRP-XL, so this effect was further investigated in relation to VASP, using the phospho-VASP (Ser239) and phospho-VASP (Ser157) antibodies to determine the mechanism of inhibition. The molecular mass of phosphorylated human VASP is ~50 kDa at Ser157, while at Ser239 it is ~48 and ~50 kDa. Phosphorylation of VASP at residue Ser239 is due to the increase in the levels of cGMP that activates PKG, while phosphorylation of VASP at residue Ser157 is due to the increase the levels of cAMP that activates PKA (Kwiatkowski *et al.*, 2003).

VASP phosphorylation was detected by Western immunoblot with these VASP antibodies. Human platelets were incubated with *S. aureus* PSMα3 (30.7 μM) for 5 minutes. Platelets treated with *S. aureus* PSMα3 were then incubated with 0.05 U/mL of thrombin or 0.5 μg/mL of CRP-XL for 90 seconds.

In this experiment, platelets treated with PGI_2 were used as a positive control for P-Ser157-VASP, whereas PAPA-NONOate was a positive control for P-Ser239-VASP. Platelets treated with CRP-XL and thrombin were used as negative controls. These data showed that 30.7μM of *S. aureus* PSMα3 able to induce VASP phosphorylation by itself (Figure 6.5, Lane 2) in human platelets and also following stimulation with 0.05 U/mL of thrombin (Figure 6.5, Lane 3) or 0.5μg/mL of CRP-XL (Figure 6.5, Lane 4). This VASP phosphorylation was at residue Ser157 (Figure 6.5), while no phosphorylation of VASP at residue Ser239 was observed (Figure 6.6, Lane 2 and Lane 4) cf PAPA-NONOate (Figure 6.6, Lane 1). Platelets were incubated with 0.25 μg/mL of PGI_2 to show phosphorylation of VASP at residue Ser157 as a positive control (Figure 6.5, Lane 6), while platelets stimulated with 0.5 μg/mL of CRP-XL (Figure 6.5, Lane 1 or Figure 6.6, Lane 3) or 0.05 U/mL of thrombin (Figure 6.5, Lane 5) did not show any signs of VASP phosphorylation.

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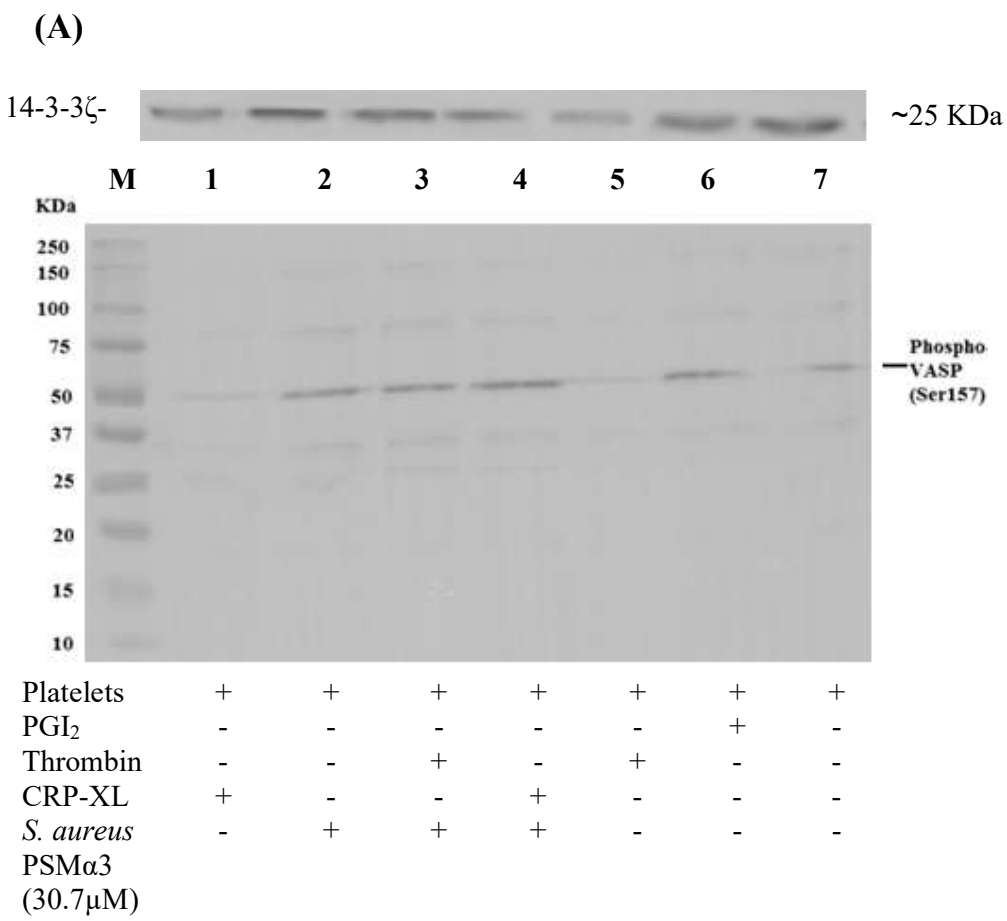

Figure 6.5: VASP phosphorylation in human platelets treated with *S. aureus* PSMα3. *S. aureus* PSMα3 induces VASP phosphorylation at residue Ser157 in human platelets. Platelets were incubated with 30.7 μM of *S. aureus* PSMα3 for 5 minutes. The PGI_2 was a positive control and CRP-XL and thrombin were negative controls. Western blot membrane with phospho-VASP (Ser157) antibody was analysed by G: BOX Chemi (n=4). Lane M, molecular weight marker and Lane 1, platelets were stimulated with 0.5 μg/mL of CRP-XL. Lane 2, platelets were incubated with PSMα3 (30.7 μM) for 5 minutes; Lane 3, platelets incubated with PSMα3 (30.7 μM) for 5 minutes and then stimulated with 0.05 U/mL of thrombin; Lane 4, platelets were incubated with PSMα3

(30.7 µM) for 5 minutes and then stimulated with 0.5 µg/mL of CRP-XL; and Lane 5, platelets were stimulated with 0.05 U/mL of thrombin. Lane 6, platelets were incubated with PGI_2; and Lane 7, resting platelets. Data showed that PSMα3 was able to induce VASP phosphorylation at residue Ser157 by itself, Lane 2, and also in presence of platelet agonists, Lane 3 and Lane 4, cf controls, Lane 6 and Lane 7. **(B)** The quantitative analysis of the phosphorylation of VASP in Western immunoblot was made using ImageJ. The intensity of signal of Ser157-VASP was normalised to the loading control signal. Then ratio of each sample was calculated. These data represent the mean ± SEM (n=4; one-way ANOVA with Tukey as a post hoc test, *P≤0.05, **P≤0.01, ***P≤0.001).

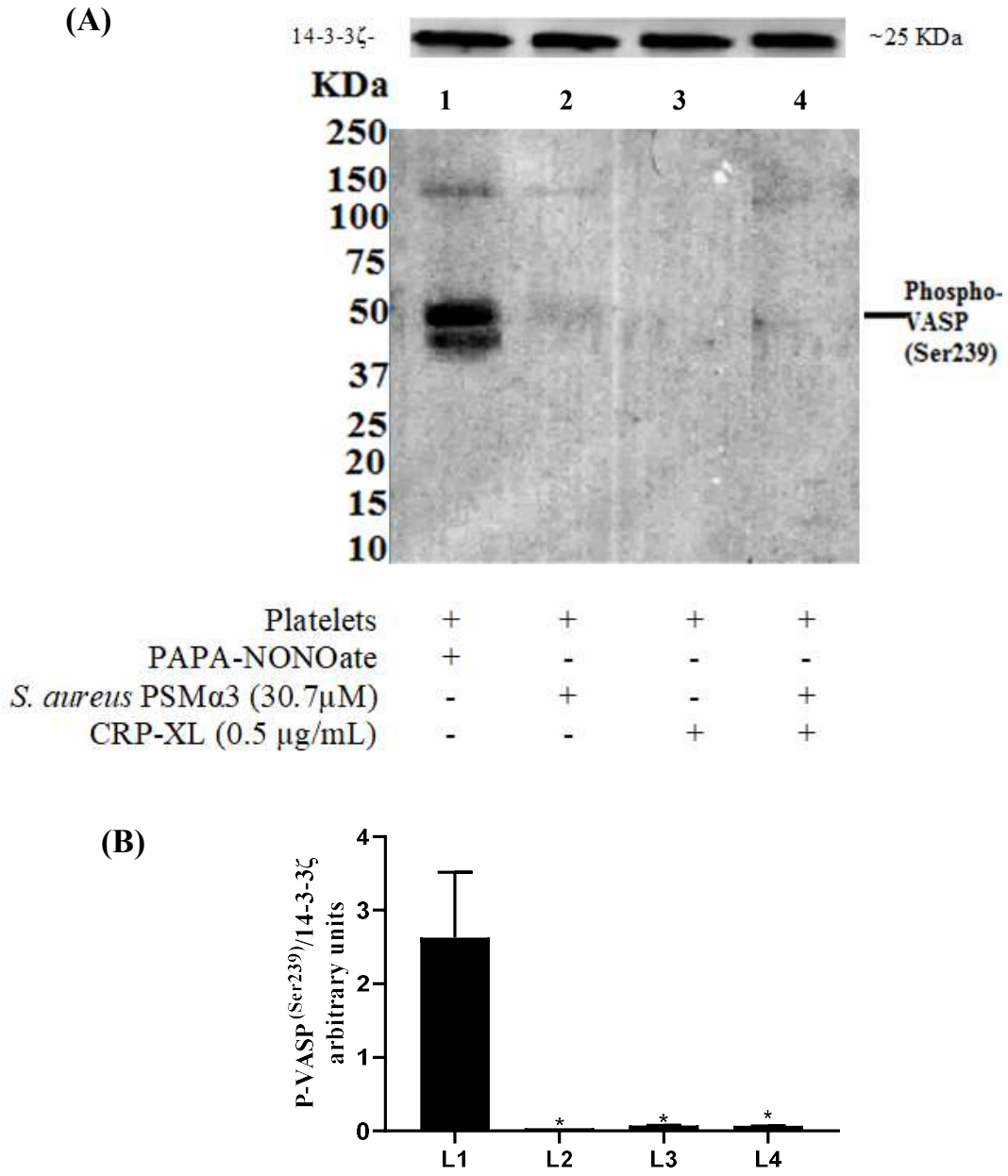

Figure 6.6: *S. aureus* PSMα3 does not induce VASP phosphorylation at residue Ser239 in human platelets. (A) A blot membrane with phospho-VASP (Ser239) antibody was performed by Typhoon FLA 9500 (n=4). The PAPA-NONOate was a positive control and CRP-XL (0.5 μg/mL) as negative control. Lane 1, platelets were pre-incubated with PAPA-NONOate; Lane 2, platelets incubated with PSMα3 (30.7 μM) for 5 minutes; Lane 3, platelets were stimulated with CRP-XL (0.5 μg/mL); and Lane 4, platelets were pre-incubated with PSMα3 (30.7 μM) for 5 minutes prior to addition of CRP-XL (0.5 μg/mL). **(B)** The quantitative analysis of the phosphorylation of VASP in Western immunoblot was made using ImageJ. The signal intensity of Ser239-VASP was normalised to the loading control signal. Then the ratio of each sample was calculated. *S. aureus* PSMα3 did not induce VASP phosphorylation at residue Ser239 in human platelets. These data represent the mean ± SEM (n=4; one-way ANOVA with Tukey as a post hoc test, *P≤0.05)

6.2.4 Measurement of intracellular cAMP in human platelets treated with *S. aureus* PSMα3

S. aureus PSMα3 has an inhibitory effect on platelet aggregation and secretion, so this effect was further tested using the phospho-VASP (Ser239) and phospho-VASP (Ser157) antibodies. These data showed that the *S. aureus* PSMα3 causes phosphorylation of VASP at Ser157 due to an increase in levels of cAMP which activates PKA. ELISA was performed to determine the concentration of cAMP in platelet lysates that were incubated with different concentrations of *S. aureus* PSMα3 with or without agonists. In this assay, PGI_2 was used as a positive control, while CRP-XL and thrombin were used as negative controls.

These data showed that the inhibitory effect of *S. aureus* PSMα3 on platelets was due, at least in part, to increasing concentrations of the cAMP that inhibit Ca^{2+} flux in platelet activation. The concentration of cAMP was increased in a dose dependent manner to *S. aureus* PSMα3 (Figure 6.7; A, B and C). Moreover, the concentration of cAMP also increased in platelets incubated with 15.38 and 30.7 µM of *S. aureus* PSMα3 in presence of 0.5 µg/mL of CRP-XL or 0.05 U/mL of thrombin (Figure 6.7 B-C). Thrombin (0.05 U/mL) and CRP-XL (0.5 µg/mL) showed decreased cAMP concentrations (Figure 6.7 B-C). This observation correlates with the pervious finding of VASP phosphorylation.

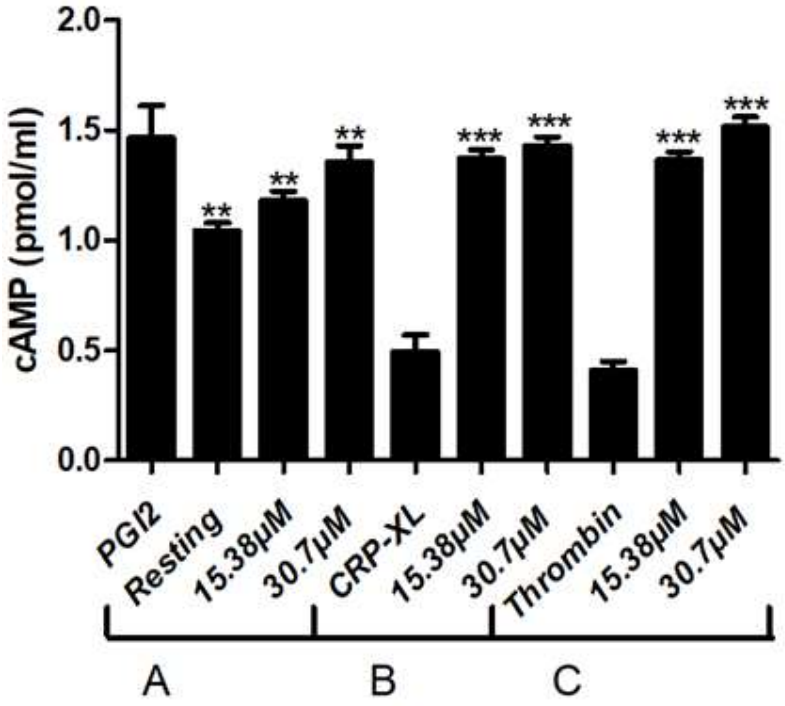

Figure 6.7: Measurement of cAMP within platelets treated with *S. aureus* PSMα3. **(A)** Platelets were incubated with 15.38 and 30.7 μM *S. aureus* PSMα3 for 5 minutes without adding CRP-XL or thrombin. After that the concentrations of cAMP were measured by ELISA. The result showed that 15.38 and 30.7 μM of *S. aureus* PSMα3 caused an increasing in the levels of cAMP more than the control. *S. aureus* PSMα3 causes enhanced levels of cAMP in platelets. **(B)** Measurement of cAMP within platelets treated with *S. aureus* PSMα3 in the presence of CRP-XL. Platelets were incubated with 15.38 and 30.7 μM of *S. aureus* PSMα3 for 5 min then with CRP-XL for 5 minutes. CRP-XL showed no effect on increase of cAMP in platelets treated with 15.38 and 30.7 μM of *S. aureus* PSMα3. **(C)** Measurement of cAMP within platelets treated with *S. aureus* PSMα3 in the presence of 0.05 U/mL of thrombin. Platelets were incubated with 15.38 and 30.7 μM *S. aureus* PSMα3 for 5 minutes then stimulated by adding thrombin for 5 minutes. The finding showed that the increased levels of cAMP in response to *S. aureus* PSMα3 were not affected by thrombin. *S. aureus* PSMα3 showed ability to elevate the levels of cAMP. These data represent ± SEM (n=4; one-way ANOVA with Tukey as a post hoc test, **P≤0.01, ***P≤0.001).

6.2.5 cAMP-dependent PKA activation in platelets treated with *S. aureus* PSMα3

Further investigation was carried out to find whether the *S. aureus* PSMα3 induced Ser157-VASP phosphorylation was related to elevated cAMP levels and also to test whether the elevation of PKA phosphorylation in treated platelets with *S. aureus* PSMα3 is cAMP-dependent or not.

In this experiment, H-89 and Rp-8-Br-cAMPS, which are two different PKA inhibitors, were used to study whether PSMα3 induces Ser157-VASP phosphorylation that was mediated by PKA. The tetrameric structure of PKA consists of two PKAr (regulatory) and two PKAc (catalytic) subunits (Potter *et al.*, 1979). Increasing levels of cAMP leads to binding of PKAr and causes dissociation of the PKA complex and release of the catalytic subunits. The PKAc can be blocked by H-89 through inhibition of ATP binding to this subunit, whereas PKAr can be inhibited by Rp-8-Br-cAMPS *via* blocking the cAMP-binding sites in this subunit.

Washed platelets were pre-incubated with 10 µM of H-89 or 200 µM of Rp-8-Br-cAMPS for 10 minutes before incubation with 30.7 µM of *S. aureus* PSMα3 for 5 minutes. Afterwards Western blotting was used for analysis. Resting platelets and PGI_2 were used as controls. The result showed that H-89 and Rp-8-Br-cAMPS strongly decreased the phosphorylation of VASP by *S. aureus* PSMα3 that showed in lane 1 and lane 2 (Figure 6.8A-B). PSMα3 induced VASP phosphorylation in platelets that were not treated with PKA inhibitors that showed in lane 3 cf controls that in lane 4 and lane 5 (Figure 6.8A-B). These data showed that *S. aureus* PSMα3 is cAMP-dependent because phosphorylation of PKA was due to elevated cAMP levels. This finding is consistent with VASP phosphorylation and increased levels of cAMP (Figure 6.8).

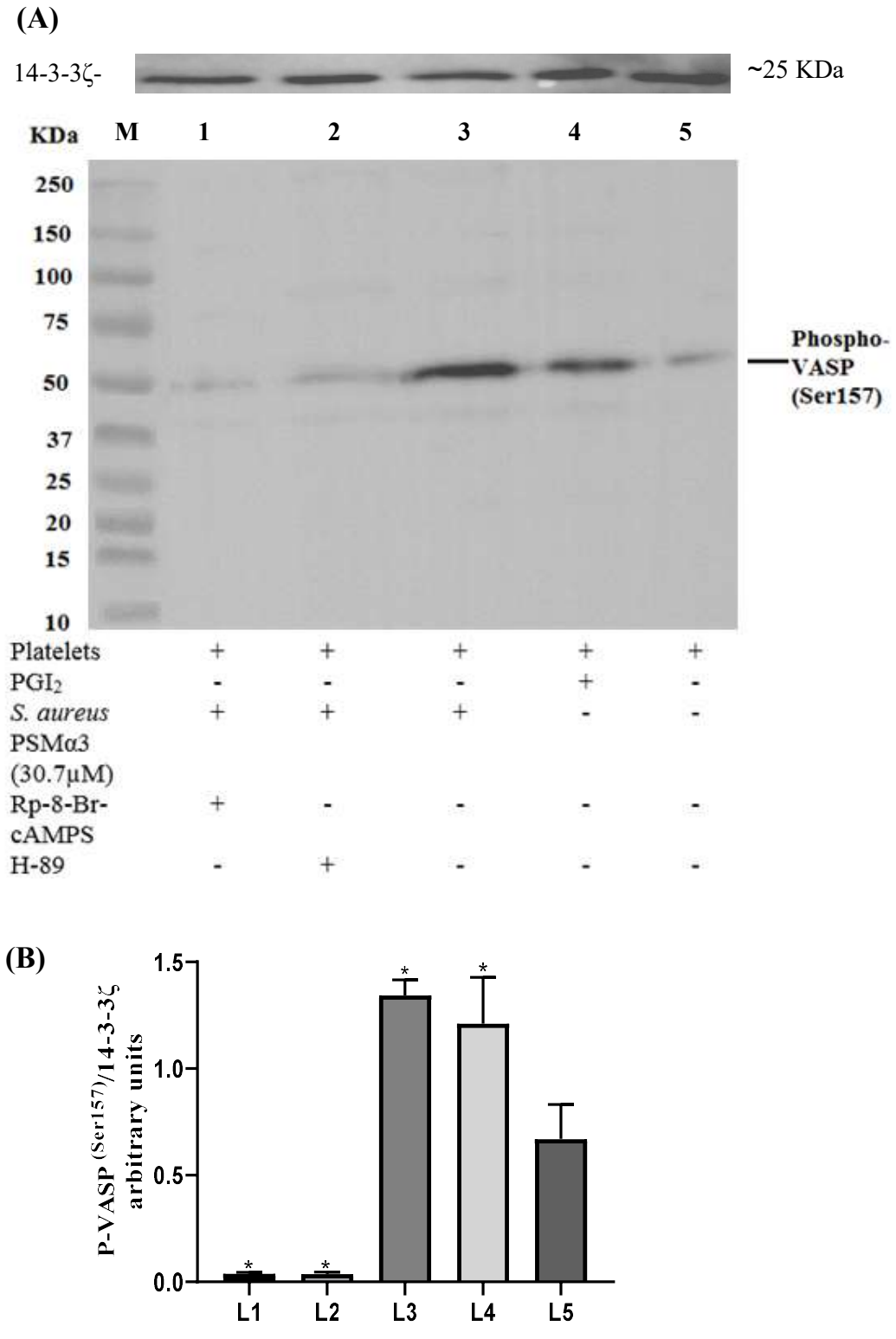

Figure 6.8: cAMP-dependent PKA activation in platelets treated with *S. aureus* PSMα3. To determine whether activation of PKA in platelets treated with PSMα3 is cAMP-dependent or not. **(A)** Western immunoblot analysis of platelets were pre-

incubated with H-89 or Rp-8-Br-cAMPS for 10 minutes prior to addition of 30.7 μM of *S. aureus* PSMα3, were incubated for 5 minutes. Resting platelets and PGI_2 were used as controls. Phospho-VASP (Ser157) antibody (Cell Signalling) was used to detect Ser157-VASP phosphorylation. Lane M, molecular weight marker. Platelets were pre-incubated with Rp-8-Br-cAMPS, Lane 1, or H-89, Lane 2, prior to addition of 30.7 μM of *S. aureus* PSMα3. Lane 3, platelets were incubated with PSMα3 (30.7 μM) for 5 minutes without pre-incubated with PKA inhibitors; Lane 4, platelets were incubated with PGI_2; and Lane 5, resting platelets. Results showed that PSMα3 did not able to induce VASP phosphorylation in the presence of PKA inhibitors, Lane 1 and Lane 2, cf Lane 3 which PSMα3 without presence these inhibitors. Based on these data, PSMα3 is cAMP-dependent PKA activation. This experiment was analysed by G: BOX Chemi (n=3). **(B)** The quantitative analysis of the phosphorylation of VASP in Western immunoblot was made using ImageJ. The signal intensity of Ser157-VASP was normalised to the loading control signal. Then the ratio of each sample was calculated. The data represent the mean ± SEM (n=3; one-way ANOVA with Tukey as a post hoc test, *$P \leq 0.05$).

6.3 Conclusions

In this chapter, the experiments were designed to gain understanding of how *S. aureus* PSMα3 regulates human platelets through signalling networks. *S. aureus* PSMα3 has an inhibitory effect on platelet aggregation and secretion. Moreover, PSMα3 inhibits intracellular calcium mobilisation (Figure 6.1 and 6.2) which is an important step in platelet activation (Rink *et al.*, 1990).

The effect of *S. aureus* PSMα3 on the upstream and downstream signalling of Ca^{2+} mobilisation was investigated through studying its effect on the activation of PLC and PKC (Figure 6.3 and 6.4). The findings showed that *S. aureus* PSMα3 prevents activation of PLC and PKC as well as through increasing the levels of cAMP, which is an inhibitory mechanism in platelets (Figure 6.7).

These findings were consistent with inhibition of calcium mobilisation, degranulation and fibrinogen binding. *S. aureus* PSMα3 modulates platelet functions, preventing platelet activation, aggregation and degranulation. The findings showed that *S. aureus* PSMα3 is able to induce phosphorylation of VASP at Serine-157 (Figure 6.5), but not at Serine-239 (Figure 6.6). The phosphorylation of VASP on Ser157 was due to an increase in the levels of cAMP that leads to activation of PKA. Raising the cAMP concentration inhibits activation and aggregation of platelets by preventing the initial Ca^{2+} flux, which blocks the activation of integrin $\alpha_{IIb}\beta_3$, and also *via* raising VASP phosphorylation leading to the prevention of formation of lamellipodia and filopodia by inhibiting actin polymerisation.

It became clear that the effects of *S. aureus* PSMα3 on human platelet a resulted from increasing the levels of cAMP. Activation of PKA *via* dissociation of PKAr-PKAc by cAMP leads to increased levels of phosphorylation of VASP. Further experiments confirmed that this interaction of *S. aureus* PSMα3 with platelets is *via* cAMP-dependent PKA activation that is due to *S. aureus* PSMα3 binding to VPAC1 receptor, which has an inhibitory function in platelets.

CHAPTER 7

General discussion, Conclusions and Future work

Chapter 7

6. General discussion, Conclusions and Future work

7.1 General discussion

7.1.1 Introduction

Platelets are involved in innate immunity, besides their primary function in haemostasis (Cox *et al.*, 2011). The functions of platelets in the immune system include recognition of invading pathogens, production of AMPs and recruitment of other immune cells to the site of infection (Cox *et al.*, 2011). Platelets express many receptors that are capable of sensing invading pathogens. For example, CLEC-2 and GPVI detect human immunodeficiency virus and hepatitis C virus, respectively. This interaction between these viruses and platelet receptors leads to platelet activation (Chaipan *et al.*, 2006; Zahn *et al.*, 2006). As a result of this activation, platelets produce PF4 that causes activation of neutrophils (Scheuerer *et al.*, 2000), which migrate to the site of infection. Consequently, activated platelets bind to these neutrophils *via* their P-selectin that binds to PSGL-1 on the surface of neutrophils (McEver *et al.*, 1997). This adhesion of activated platelets to neutrophils induces phagocytosis of platelets, eliminating the infection by clearance of platelets from the blood circulation by the liver and spleen (Grozovsky *et al.*, 2010). Thus, the platelet has acted against the virus, before it can be eliminated by a phagocyte.

Moreover, platelets release Tβ-4 and regulated on activation normal T expressed and secreted (RANTES), which are AMPs that are able to attack pathogens (Tang *et al.*, 2002). For instance, Tβ-4 has a strong effect on *Escherichia coli* and *S. aureus* (Tang *et*

al., 2002). RANTES kills fungal pathogens like *Candida albicans* and *Cryptococcus neoformans* (Tang *et al.*, 2002).

S. aureus is a common cause of bacteraemia (Naber, 2009) that leads to severe invasive infections such as sepsis and IE (Graham *et al.*, 2006). During bacteraemia, platelets are considered the first cells of the host that encounter the invading pathogens (Cox *et al.*, 2011) and *S. aureus* interacts with them by producing surface proteins and toxins that affect physiological functions (Fitzgerald *et al.*, 2006). Extracellular proteins of *S. aureus* have been studied for their abilities to affect platelet activity (Fitzgerald *et al.*, 2006; Hartleib *et al.*, 2000; Waller *et al.*, 2013). PSM toxins play important roles in staphylococcal infections. PSMs are able to stimulate neutrophils at nanomolar concentrations and lyse them at micromolar concentrations (Wang *et al.*, 2007), but PSMs do not lyse macrophages at micromolar concentrations (Schreiner *et al.*, 2013).

The role of PSMα3 and other types of PSMs have been not evaluated in the progression of IE. In previous *in vivo* studies (Cheung *et al.*, 1994; Huseby *et al.*, 2010; Pragman *et al.*, 2004) however, it has been shown that cytolysins and superantigens have a major role in IE (Cheung *et al.,* 1994; Huseby *et al.,* 2010; Pragman *et al.,* 2004). PSMα3 and other PSMs are important in the development of lethal sepsis (Spaulding *et al.*, 2012). Neutralizing PSMs causes delayed progression of lethal sepsis (Spaulding *et al.,* 2012). However, this study did not show that PSMs play a role in IE because mutating PSM genes did not affect valve lesions of the heart in a rabbit model of experimental endocarditis (Spaulding *et al.,* 2012). PSMα3 did show an ability to limit *in vitro* thrombus formation (Figure 3.6) by inhibition of platelet activation (Figures 3.2-3.5). This mechanism could therefore contribute to spread of *S. aureus* within the host. For

example, IE is commonly a biofilm-associated infection (Jung *et al.*, 2012). PSMs have been shown to enable spreading of bacteria from colonized catheter in a model of biofilm-associated infection (Periasamy *et al.*, 2012).

Obviously, PSMs are not the only virulence determinants that are secreted by *S. aureus*. The bacterium produces many toxins such as PVL and α-toxin. These are involved in the pathogenesis of *S. aureus* and contribute to different pathologies in humans such as skin and soft tissue infections. For example, PVL is cytolytic towards neutrophils (Genestier *et al*., 2005; Löffler *et al.,* 2010) and contributes to abscess formation in the skin (Badiou *et al.*, 2008; Badiou *et al.*, 2010; Lipinska *et al.*, 2011; Panton *et al.*, 1932). α-toxin is a virulence determinant that plays an important role in abscess formation (Kobayashi *et al.*, 2011). PSMs work with other virulence determinants in a synergistic fashion. For example, PSMs work in concert with PVL to reach the maximal pathogenesis during skin or soft tissue infections (Hongo *et al.*, 2009). In addition, the cytolytic effect of PVL against neutrophils is enhanced by PSMα3 (Hongo *et al.*, 2009). PSMα3 might work alongside the number of other virulence factors of *S. aureus* that activate or impair platelet function. For example, PSMα3 works with α-toxin to enhance the adherence of *S. aureus* to the endothelium at wound sites. α-toxin-ADAM10 complex causes GPVI proteolysis which blunts activation of $\alpha_{IIb}\beta_3$ that is essential for aggregation (Powers *et al.,* 2015). PSMα3 inhibits activation of $\alpha_{IIb}\beta_3$ (Figure 3.7), therefore, platelets are unable to launch fibrinogen bridges between them, resulting in failure to form a plug at the injured endothelium (Tronik-Le Roux *et al.,* 2000). Inhibition of platelet aggregation would be beneficial for a pathogenic bacterium to inhibit wound healing and facilitate access to bloodstream. Upon attachment of bacteria to the endothelium of a heart valve, α-toxin triggers platelet aggregation cause an enlargement of the valvular vegetation (Fowler *et al.,* 2005). The ability to determine the actual, relative *in vivo* roles of virulence factors, in concert with one another, is currently not technically possible and

requires a fuller understanding of the array of virulence factors produces, their expression profiles and appropriate animal models. In this thesis, the effects of *S. aureus* PSMα3 on platelets were investigated.

7.1.1.2 *S. aureus* molecules and their effects on platelets

S. aureus produces molecules such as SpA, LTA, FnBPA, FnBPB and ClfA that can affect platelet function positively or negatively (Fitzgerald *et al.*, 2006; Hartleib *et al.*, 2000; Waller *et al.*, 2013). The interaction occurs between these molecules and platelets, either by binding to platelet receptors or to bacterial proteins that are covered with plasma proteins (Loughman *et al.*, 2005).

Different *S. aureus* molecules have been shown to affect platelet function, either by causing activation or inhibition. SpA binds to vWF that leads to activation of platelets (Hartleib *et al.*, 2000). FnBPA, FnBPB and ClfA indirectly bind to platelets causing them to aggregate through the formation of a link between fibrinogen or fibronectin, acting as a bridging molecule to integrin $\alpha_{IIb}\beta_3$ (Fitzgerald *et al.*, 2006). On another hand, LTA causes platelet inhibition via PafR (Waller *et al.*, 2013).

7.1.2 Effects of *S. aureus* PSMα3 on platelets

7.1.2.1 Inhibition of platelet activation by *S. aureus* PSMα3

In this work, *S. aureus* PSMα3 inhibited platelet activation mediated by physiological platelet agonists and the pathogen. The ability of *S. aureus* PSMα3 to reduce platelet aggregation occurred through impairment of PLC and PKC activation (Figures 6.3-6.4)

that inhibited mobilisation of Ca^{2+} (Figures 6.1-6.2) and $\alpha_{IIb}\beta_3$ integrin activation (Figure 3.7). As a result of this impairment of PKC, P-selectin exposure was inhibited (Figure 3.8) that is required for recruitment and stimulation of nearby platelets (McEver, 2002).

Furthermore, thrombus volume was reduced in platelets treated with *S. aureus* PSMα3 (Figure 3.6). This effect of *S. aureus* PSMα3 on thrombus formation is due to the failing of platelet integrin $\alpha_{IIb}\beta_3$ to bind fibrinogen (Figure 3.7). As a result of this failure of fibrinogen binding to activated integrin $\alpha_{IIb}\beta_3$ leads to the inhibition of cytoskeletal rearrangements and outside in signalling. Thus inhibiting of clot retraction and platelet spreading (Figures 3.10-3.12) (Bennett, 2005; D. Lee *et al.*, 2012). These inhibitory effects of *S. aureus* PSMα3 on platelets were observed with sustained increase of cAMP levels (Figure 6.7).

cAMP is an important secondary messenger that plays a role in the balancing of immune cells between activation and inhibition (Borger *et al.*, 2000; Johansson *et al.*, 2004). Increased intracellular levels of cAMP leads to PKA activation that maintains platelets in a resting state (Skalhegg *et al.*, 2000). Activation of the cAMP/PKA pathway can have a strong impact on the function of innate immune cells, through reduction of pro-inflammatory mediators, suppressing the proliferation of immune cells and thus microbial clearance. The level of cAMP controls the balance of production of pro-inflammatory and anti-inflammatory mediators in immune cells such as neutrophils, macrophages and dendritic cells (van der Pouw Kraan *et al.*, 1995; Aronoff *et al.*, 2006). Suppression of these mediators leads to reduced responses of immune cells to infection.

Increased intracellular levels of cAMP leads to the release of anti-inflammatory mediators such as interleukin-10 from immune cells (Aronoff *et al.*, 2006; Gasperini *et al.*, 2002). Activation of PKA due to increased intracellular levels of cAMP also inhibits macrophage inflammatory protein-1a and -1b (Aronoff *et al.*, 2006). Elevation of intracellular cAMP can suppress the proliferation of immune cells such as T-cells through inhibiting the production of cytokines like IL-2 and IL-5 (Bryce *et al.*, 1999).

7.1.2.2 *S. aureus* PSMα3 modulates platelets through VPAC1 receptor

FPR2 was identified as the principal receptor for *S. aureus* PSMα3 in neutrophils by Kretschmer *et al*., (2010). Detection of *S. aureus* PSMα3 by FPR2 in platelets had not been established until this work. FPR2 is a seven transmembrane protein like all GPCRs and is expressed on both neutrophils and platelets (Czapiga *et al.*, 2005; Migeotte *et al.*, 2006). However, a principal difference is in its ability to detect its natural ligands such as Annexin A1 (ANXA1) and N-formylated peptides (Bena *et al.*, 2012). ANXA1 is an endogenous protein that interacts with FPR2 on neutrophils and subsequently leads activate these cells (Bena *et al.,* 2012; Vital *et al.*, 2016). While FPR2 on platelets is unable to detect and interact with ANXA1 (Vital *et al.,* 2016). Moreover, mouse Fpr2 is an ortholog of human that shares its binding properties with FPR2. Mouse Fpr2 is also unable to detect most N-formylated peptides (He *et al.*, 2013).

FPR2 was detected in human platelets by Western immunoblotting (Figure 4.1). This receptor appeared in a punctate arrangement on the surface of the platelets (Figure 4.2). FPR2 function was tested by use of a specific agonist and antagonist. Agonist MMK-1 induced platelet aggregation (Figure 4.3), whereas WRW4 caused inhibition of platelet

aggregation mediated by MMK-1 (Figure 4.4). These data showed that FPR2 is a functional receptor in platelets.

The action of *S. aureus* PSMα3 was not abrogated by WRW4, a selective FPR2 antagonist (Figure 4.4), suggesting that the effects of PSMα3 on platelets were independent of FPR2. VPAC1 receptor was identified by mass spectrometry analysis as likely to be the major surface protein that interacts with *S. aureus* PSMα3 (Figure 4.5).

Increased levels of cAMP inside platelets treated with *S. aureus* PSMα3 was mediated by VPAC1 (Figures 4.6-4.7), a G protein coupled receptor (Gs)-GPCR (Vaudry *et al.*, 2000). Interaction of VPAC1 with its agonists causes activation of adenylyl cyclase. This activation leads to an increase in the level of cAMP and causes activation of PKA (Vaudry *et al.*, 2000). In platelets, VPAC1 receptor works as a negative regulator of platelet activation (Freson *et al.*, 2004; Freson *et al.*, 2008) *via* activation of PKA, thereby preventing any further platelet activation and aggregation (Peeters *et al.*, 2010).

Structurally, *S. aureus* PSMα3 is similar to other types of α-PSMs; α-PSMs share some common properties. First of all, the length of these peptides is ~20 amino acids and they have obvious α-helical structures. All of them carry N-formyl methionine and have a central α-helix with tight bends at the termini. However, there are some differences in the position of hydrophilic and hydrophobic amino acids, characteristic of residue charge distribution and amphipathic α-helical in these peptides. Each α-PSMs peptide has one hydrophobic side and one hydrophilic side, but there are in differences in the arrangement of hydrophilic and hydrophobic amino acids. For example, PSM's α1 and α2 have identical hydrophobic characters, whereas PSMα3 has slightly more hydrophobic regions.

This peptide has the largest hydrophobic and hydrophilic moment among these peptides (Towle *et al.,* 2016). Also there is a different arrangement of residue charges in these peptides. A positively charged amino acid residue which interacts with a negatively charged residue may create a salt bridge (Smith *et al.,* 2017) and is thus important for stabilization of α-helix formation. PSMα3 has the largest number of amino acids with positive or negative charges connected by ionic bonds (Towle *et al.,* 2016). In PSMα3, two salt bridges appear between residues Glu^{3} and Lys^{6} and residues Asp^{13} and Lys^{17}. While there is a single salt bridge present between residues Lys^{12} and Glu^{16} in PSMα1 (Laabei *et al*., 2014). The amphipathic helix runs from residue 2 to 20 in PSMα3, whereas it runs from residue 2 to 19 in PSMα1.

Indeed, the properties of PSMα3 that have been published by Laabei et al. (2014) were found to be in agreement with structural characteristics of PSMα3 (Figure 5.1-5.2). Bioinformatic analysis indicated that phenylalanine and methionine residues in PSMα3 may play an important role in interaction with VPAC1 (Figure 5.7) which is in agreement with a previous study that showed presence of N-formyl methionine is essential for the interaction of α-PSM peptides with host receptors (Kretschmer *et al.,* 2010). Phe^{1}, Met^{2} and Phe^{4} residues of PSMα3 are predicted to be essential for the interaction with VPAC1 (Figure 5.7). These residues directly interact with His^{47} and Leu^{51} residues of N-terminal domain of VPAC1 receptor (Figure 5.7). The N-terminal domain of VPAC1 receptor is a common binding site for PSMα3 and VIP (Couvineau *et al.*, 2012). These residues in the N-terminal domain are involved in the activation of this receptor (Tan *et al.*, 2006). Activation of VPAC1 causes activation of cAMP/PKA pathway. Of note, the ability of other α-PSMs to engage with VPAC1 may be open to question because of these

difference among these peptides, as mentioned above, which may influence the interaction of peptides with VPAC1.

To follow up on these bioinformatic studies, the inhibitory effects of *S. aureus* PSMα3 on platelet function were further investigated in order to determine the mechanism(s). Platelets treated with *S. aureus* PSMα3 showed increased intracellular levels of cAMP (Figure 6.7). Platelets pre-incubated with *S. aureus* PSMα3 then treated with thrombin or CRP-XL showed increased levels of intracellular cAMP and activation of PKA (Figures 6.7-6.8). On the other hand, activatory pathways such as PLC and PKC were inhibited (Figures 6.3-6.4). This result was consistent with inhibition of calcium mobilisation in platelets treated with *S. aureus* PSMα3 (Figures 6.1-6.2). Activation of cAMP/PKA pathway by *S. aureus* PSMα3 (Figure 6.8) prevents platelet activation. This finding was consistent with bioinformatic studies (Figure 5.6-5.7). A model was proposed for inhibition of platelet activation by *S. aureus* PSMα3 (Figure 7.1). Activation of VPAC1 receptor by PSMα3 leads to reduced activation and aggregation of platelets, which is consistent with reports from Freson *et al*., (2004) and Kis *et al*., (1999) that showed activation of VPAC1 prevents platelet aggregation by the same mechanism.

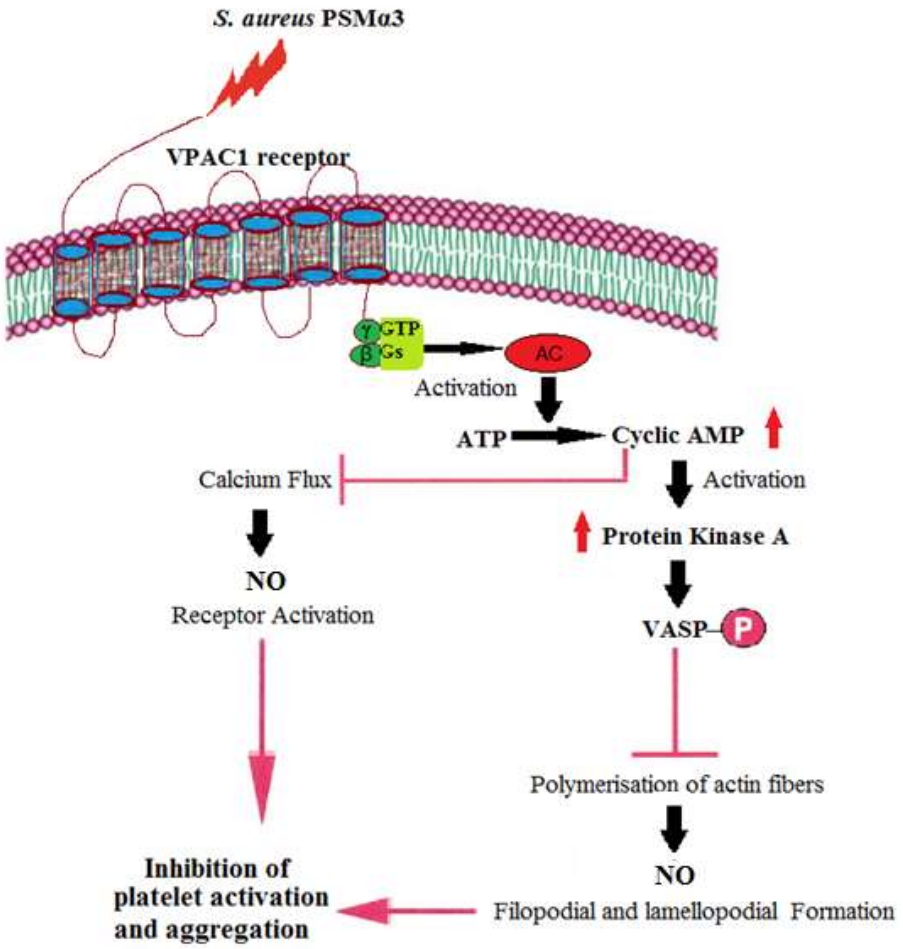

Figure 7.1: Proposed model of inhibition of platelet activation and aggregation by *S. aureus* PSMα3. *S. aureus* PSMα3 binds to VPAC1 receptor that leads to an exchange of nucleotides, followed by dissociation of Gsα of VPAC1 receptor which leads to activation of adenylate cyclase (AC). Then increasing levels of cAMP due to activation of AC causes activation of protein kinase A (PKA) and inhibits the calcium flux. Inhibition of the calcium flux prevents activation of integrin $\alpha_{IIb}\beta_3$ and other receptors. PKA causes phosphorylation of VASP that prevent actin polymerization and therefore there is no formation of filopodia and lamellopodia. Consequently, *S. aureus* PSMα3 inhibits platelet functions: aggregation, fibrinogen binding, granules release and calcium mobilisation.

7.1.2.3 Inhibition of platelet activation by *S. aureus* PSMα3; significance in interaction of host-pathogen.

Interaction of *S. aureus* with platelets contributes to the pathogenesis of bacteria inside the host, particularly in IE (Fitzgerald *et al.*, 2006). During IE, the formation of a vegetation is proposed to occur via interaction of platelets with circulating *S. aureus* at the site of injury (Werdan *et al.*, 2014). *S. aureus* releases molecules that are able to bind to platelets and modulate their function (O'Brien *et al.*, 2002). The capacity of *S. aureus* to be inside a platelet plug may allow the organism to remain at the site of infection and to resist the shear forces of flowing blood (Pawar *et al.*, 2004). *S. aureus* molecules like LTA and Efb might control a growing thrombus by inhibition of platelet activation (Waller *et al.*, 2013; Shannon *et al.*, 2004). This mechanism could therefore contribute to *S. aureus* growth inside the host and dissemination within the host.

S. aureus PSMα3 hijacks host cell processes to raise cAMP levels to deactivate the immune response of the host, consequently inhibiting pro-inflammatory cytokines and enhancing anti-inflammatory mediators (Deplanche *et al.*, 2016; Schreiner *et al.*, 2013). For instance, *S. aureus* PSMα3 suppresses the release of IL-6, TNF and IL-12, which are pro-inflammatory cytokines, from DCs (Schreiner *et al.*, 2013), while elevation of levels of anti-inflammatory IL-10 is observed with DCs treated with PSMα3 (Schreiner *et al.*, 2013). Furthermore, *S. aureus* PSMα3 impairs production of cytokine IL-32 from mammary epithelial cells that affects maturation of DCs (Deplanche *et al.*, 2016).

Suppression of IL-32 expression by pathogens leads to dysfunction in adaptive immune cells. For example, DCs treated with PSMs show decreased ability to activate T-cells (Schreiner *et al.*, 2013). Deactivation of the immune response from T cells leads to a

decreased ability to clear an infection (Schreiner *et al.*, 2013). The anti-inflammatory action of PSMs occurs by increasing levels of expression of IL 10, leading to inhibition of inflammatory responses, which assists the spread of pathogens inside the host (McGuirk *et al.*, 2002).

Accordingly, *S. aureus* PSMα3 is believed to play a significant role in host-pathogen interactions. Therefore, this action of *S. aureus* PSMα3 could be important for pathogenesis of *S. aureus* through inhibition of platelet activation and dissemination of the pathogen within the host.

Many pathogens use cAMP of the host as a mechanism for survival and persistence of a chronic infection within the host. Maintenance of chronic infection occurs through successful evasion of the responses of immune cells and causes dysfunction of the immune system. Several reports show that many pathogens can stimulate cAMP production inside host cells for establishing an infection (Vanden Broeck *et al.*, 2007; Katada *et al.*, 1982).

For example, *Bordetella pertussis* produces pertussis toxin (PT) which plays an important role during whooping cough infection (Carbonetti *et al.*, 2003). This toxin causes elevation of intracellular cAMP in macrophages and therefore leads to impaired phagocytosis (Katada *et al.*, 1982) and reduced production of superoxide (Hiemstra *et al.*, 1992). In addition to the effects of PT, signalling to neutrophils to migrate to the site of infection is inhibited by this toxin and leads to failure to clear this pathogen (Kirimanjeswara *et al.*, 2005). These inhibitory effects of PT work through catalysing

ADP ribosylation of the Gαi subunit of GPCR, that increases levels of intracellular cAMP within the immune cell (Katada *et al.*, 1982).

Vibrio cholera is another example of how a pathogen can use cAMP of host cells for persistence during cholera infections. The bacterium produces cholera toxin (CT) that catalyses the ADP ribosylation of Gαs subunits of GPCR (Vanden Broeck *et al.*, 2007). The result of this catalysing the ADP ribosylation leads to activation of AC by binding to Gαs subunits of GPCR (Vanden Broeck *et al.*, 2007) which thus leads to raised levels of intracellular cAMP in phagocytes (Vanden Broeck *et al.*, 2007). Inhibition of phagocytic function is a consequence of the rising levels of intracellular cAMP by CT (Niemialtowski *et al.*, 1993).

Generally, pathogenicity of CA-MRSA infections is strongly related to secretion of PSMs e.g. PSMα3, principally in skin or soft tissue infections (Wang *et al*., 2007). These toxins may play an enhanced role in the progression of these infections by eliciting cytotoxic and pro-inflammatory effects which can damage epithelial cells and neutrophils (Cassat *et al*., 2013; Cheung *et al*., 2012; Giese *et al*., 2011; Wang *et al*., 2007). As a consequence of this, skin and soft tissue infections can progress to wound infections that are associated with bleeding. Following bleeding, circulating platelets adhere to the wound and start to aggregate to form a stable plug that seals the wound. Wound healing requires platelets to form a platelet plug and release VEGF and PDGF from their α granules, which stimulate wound repair (Barrientos *et al.*, 2008). However, this process might be more difficult in presence of bacteria or their products e.g. PSMα3. Interactions of PSMα3 with platelets could influence skin or soft tissue infections through inhibition of platelet functions. Inhibition of platelet aggregation (Figure, 3.2-3.5),

granule release (Figure 3.8) and clot retraction (Figure 3.10) by PSMα3 is presumed to be beneficial for bacteria to inhibit wound healing. This effect would assist in increasing the size of the wound and also could assist in spreading the bacterium within the hosts body causing a systemic infection (Athanasopoulos *et al.*, 2006; Shannon *et al.*, 2005).

PSMα3 has a major effect on e.g. progression of sepsis or soft tissue infections (Wang *et al*., 2007). Mice were used as a sepsis model and were infected with the wild-type pathogen, which displayed greater progression to the moribund state than mice infected with isogenic *psmα* mutant (Wang *et al.*, 2007). Moreover, deletion of the *psmα* causes loss the potency of the pathogen in formation of a subcutaneous abscesses (Wang *et al.*, 2007). Accordingly, this work could be further developed to provide immunization against CA-MRSA infections. PSMα3 could be a target for the development of anti-staphylococcal therapy. There are a number of advantages of targeting the PSMs e.g. PSMα3; (i) PSMα3 is the most cytolytic peptide over other PSMs peptides. (ii) PSMs form a major component of exoprotein repertoire of CA-MRSA. (iii) PSMs play the main role in different parts of *S. aureus* infection. (iv) There are a few difference in sequences of amino acid of PSMs among *S. aureus* strains (Wang *et al.*, 2007). Thus, deactivation of PSMα3 activity could reduce the potency of CA-MRSA towards establishing disease e.g. soft tissue infections.

However, there would be some of the challenges in vaccinating against PSMs. First, immunization against these toxins would likely require high concentration of antibodies, because CA-MRSA produces high concentrations of PSMs. Secondly, the role of PSMs in the interaction between the pathogen and professional host phagocytes is well established, and vaccination would be unlikely to be able to inhibit this interaction

(Surewaard *et al.*, 2012). Finally, *S. aureus* produces a large number of toxins that play different roles in infections. Thus, making it difficult to generate antibodies reach to all the different type of toxin produced.

7.2 Conclusions

To conclude, *S. aureus* PSMα3 showed an effect on the physiological functions of platelets, inhibiting their aggregation and granules release. *S. aureus* PSMα3 had an inhibitory effect in a dose-dependent manner on different agonist-induced platelet aggregation.

This inhibition was due to interaction of *S. aureus* PSMα3 with VPAC1 receptor. This interaction enhances the inhibitory mechanism in platelets *via* increasing the levels of cAMP, followed by activation of PKA that prevents any further activation of platelets.

7.3 Future work

S. aureus PSMα3 showed an ability to inhibit platelet activation through VPAC1 receptor. Thus, further investigations are needed to confirm this inhibition occurs solely *via* VPAC1 receptor by using platelets obtained from VPAC1 receptor-knockout mice (Fabricius *et al.*, 2011). These investigations should include aggregation assays, measurement of cAMP and detection of VASP phosphorylation by Western blotting.

In addition, future work is needed in *in vivo* conditions because previous experiments were carried out *in vitro* conditions. Intravital microscopy will be a powerful tool to examine the effect of *S. aureus* PSMα3 *in vivo* (Rosen *et al.*, 2001). This model will study thrombus formation in *in vivo* VPAC1 receptor-*knockout* mice and will be compared to the *in vitro* thrombus formation under flow data. Moreover, mouse models of infection/disease such as mouse-tail bleed assay will assist in studying the impact of *S. aureus* PSMα3 on haemostasis (Getz *et al.*, 2015).

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