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1. Introduction

Endocarditis is an inflammation of the lining of the heart and valves. It can be due to a non-infectious cause (Asopa et al., 2007) but when the inflammation is associated with an infection, usually bacterial, it is known as infective endocarditis (IE) and is characterized by the development of a large septic thrombus on one of the cardiac valves (Beynon et al., 2006; Moreillon and Que, 2004). As this thrombus grows, it can lead to valve failure or may fragment forming a septic embolus that is associated with high mortality if the target of the embolus is the brain, heart or lung (Homma and Grahame-Clarke, 2003). Untreated the mortality is very high and even with aggressive therapy with antibiotics and valve replacement surgery there is a significant mortality. Primarily the disease is due to the formation of a platelet-bacteria thrombus on a cardiac valve and this review will look at the interaction between bacteria and platelets within the context of endocarditis.

2. Traditional role of platelets in thrombosis

Platelets are anucleate fragments of megakaryocytes and their primary role is in haemostasis. Damage to the endothelium surrounding blood vessels leads to the exposure of the sub-endothelial layer that is rich in collagen and immobilised plasma proteins such as von Willebrand factor (vWf). Platelets bind to the newly exposed collagen and become activated. Many blood vessels, such as the coronary arteries are high shear vessels and the blood is flowing too fast to allow the platelets to bind to the collagen. However, under these high shear conditions immobilised vWf can interact with platelet GPIb, which slows down the platelets allowing them to subsequently interact with collagen. Once activated platelets aggregate to form a thrombus which the damaged blood vessel. GPIIb/IIIa mediates platelet aggregation by binding plasma fibrinogen, which as a divalent protein can bind to two separate GPIIb/IIIa molecules. As a result, activated platelets are cross-linked by fibrinogen, which forms the aggregates that seal the damaged vessel.

Platelets are highly responsive and can be activated by many different agonists. As well as two different collagen receptors ($\alpha2\beta1$ and GPVI) and two ADP receptors P_2X_1 and P_2Y_{12}, a serotonin receptor, an adrenergic receptor and a thromboxane A_2 receptor there are also three thrombin receptors (Protease-activated receptor (PAR)-1, GPIbα and PAR-4). Platelet

activation is dependent on the sum of the signals from all of these receptors and not on any specific receptor. Signalling from these receptors is either phospholipase A_2/cyclooxygenase (COX)-mediated, resulting in the generation of thromboxane A_2, or protein kinase C-mediated. In contrast, the generation of intracellular cAmp in response to prostacylin binding to its receptor acts to inhibit platelet activation.

Aside from their ability to adhere to sites of damage and to form aggregates, platelets also secrete their granule contents in response to activation. Platelets contain three different granules: α-granules, dense granules and lysosomes. Dense granules are rich in small molecules especially ATP/ADP and serotonin, α-granules contain numerous plasma proteins typically associated with haemostasis and the lysosomes contain acid hydrolases. These secreted products act to enhance haemostasis as ADP activates other platelets, serotonin causes vasoconstriction and the secreted proteins support platelet aggregation.

3. The role of platelets in innate immunity

The biological role of platelets is not confined to haemostasis as platelets also play an important role in the innate immune system (Cox et al., 2011; Semple and Freedman, 2010). Platelets are ideally suited to this as they are present at very high concentration in the blood and are the first responders to any damage to the vasculature, which is the primary mechanism by which pathogens gain entry to the blood. To facilitate this, platelets contain surface receptors that allow it to respond to pathogens. Platelets contain pattern recognition receptors, especially Toll-like receptors (TLRs). These and other receptors (see below) allow platelets to respond to pathogens. As with other platelet agonists, platelets respond to pathogens by adhering to them and subsequently become activated leading to thrombus formation and secretion. In the context of the innate immune system, secretion is the primary response. Activated platelets secrete anti-microbial peptides that act to kill bacteria (Mercier et al., 2004). They also secrete cytokines such as CD40L and RANTES (Antczak et al., 2010) that act to recruit and activate a variety of immune cells to deal with the invading microorganism. Thus, platelets play a key role in the innate immune system where they directly act to kill bacteria as well as coordinating the response of the immune system to the pathogens.

4. The role of platelets in infective endocarditis

IE arises when the bacteria subvert the platelet response to infection and as a result, platelets become part of the pathogenic process. In IE the platelets interact with the bacteria and become activated. They secrete anti-microbial peptides but the bacteria are resistant. Once activated the platelets aggregate resulting in the formation of a platelet-bacteria aggregate. Not only are the bacteria resistant to the anti-bacterial peptides (Bayer et al., 1998; Fowler et al., 2000), they become surrounded by platelets and are able to evade immune surveillance. To further complicate matters even if the bacteria are susceptible to antibiotics, many antibiotics have poor penetration into the thrombus despite adequate plasma levels making treatment more difficult.

While the list of pathogens that have been known to cause IE is long (Baddour et al., 2005), the vast majority of cases with an identified pathogen are due to Staphylococci (primarily *S. aureus*) or Streptococci (primarily *S. sanguinis and S. oralis*). However, around 25% of IE cases

are culture negative with no bacteria isolated from the blood (Naber and Erbel, 2007). There are a number of reasons for the failure to culture any organisms such as commencement of antibiotic therapy prior to obtaining the blood sample, infection with a fastidious bacterium or a non-bacterial (e.g., fungal) endocarditis. In this review we will focus on the mechanism of platelet activation by Staphylococci and Streptococci as not only are they the dominant species involved in IE but they are also the best studied. While even different strains of a bacterial species differ in their ability to interact with platelets some general principles can be seen which is important in devising new treatment strategies.

5. Platelet-bacterial interactions: General observations

There are three different types of interaction between platelets and bacteria. The first is an inherent ability of platelet receptors to recognise bacterial surface components. Alternatively, plasma proteins can bind to bacteria and these proteins can in turn bind to a platelet receptor. Typically these are acute phase reactants such as fibrinogen and complement. A third mechanism for interacting with platelets is the secretion of bacterial products or toxins that interact with platelets. The binding of platelets to bacteria either through a direct interaction or via a bridging protein can mediate platelet adhesion and/or platelet activation. Interactions with bacterial toxins leads to either platelet lysis or platelet activation. Typically bacterial proteins that mediate adhesion are distinct from those that mediate aggregation. Thus, bacteria can support platelet adhesion and/or trigger platelet activation. During sepsis the primary interaction between platelets and bacteria is platelet activation and is mediated by both toxin secretion and a direct interaction with the bacteria. However, infective endocarditis is a focal infection of a damaged heart valve. The initial step is mediated by adhesion to the damaged valve as well as subsequent adhesion of platelets to the immobilised bacteria. This adhesive interaction is important in ensuring that the bacteria and thrombus remain attached to the valve despite the presence of turbulent flow conditions. For IE to develop the initial adhesive interaction must be followed by activation of the platelets leading to platelet recruitment and growth of the thrombus. Most bacteria can interact with platelets through multiple mechanisms making it difficult to identify the roles of the different proteins (both bacterial and platelet) and is further complicated by interactions that are not only species-specific but strain-specific as well.

6. Platelet-bacterial interactions: The Staphylococcus

Regardless of the modern advances in antimicrobial therapy and surgical intervention *Staphylococcus aureus* is still the most frequent etiologic microorganism found in Infective endocarditis (Rasmussen et al., 2011). Its interaction with platelets is well characterised. Much of the investigations to date have focused on two separate but related features of this relationship; 1) toxins and 2) cell wall protein mediators of platelet activation (Table 1).

S. aureus is known to secrete several extracellular toxins. Alpha (α)-toxin is a 34 kDa toxin composed of 293 amino acids (Bernheimer, 1965). It is produced by almost all strains of *S. aureus*. Its expression is accessory-gene regulon (agr)-regulated and is secreted into the extracellular environment as a monomeric water soluble protein (Ikigai and Nakae, 1985). The toxin disrupts the cell membranes by binding to the lipid bilayer, forming an oligomeric structure that forms a water filled transmembrane pore (Valeva et al., 1996). Studies have demonstrated that the toxin has primarily two modes of interaction with host

cells. These include binding specifically to the host target at low concentrations and non-specific adsorption to host target cell membranes at higher concentrations (Hildebrand et al., 1991).

Siegel and Cohen were the first to demonstrate that addition of α-toxin to human platelet-rich plasma induced platelets to undergo shape change and aggregation (Siegel and Cohen, 1964). In this study the authors demonstrated that platelets leaked their intracellular ions NAD^+, K^+ and ATP but not protein, into the surrounding environment thus concluding that the platelets were not being lysed. Additional studies demonstrated that when platelets were treated with α-toxin it caused them to swell but there were no clear signs of platelet lysis by electron microscopy (Bernheimer and Schwartz, 1965; Manohar et al., 1967). Further investigations into the molecular mechanism through which platelets became activated, found that pore formation gave rise to an increase in intracellular calcium concentration (Arvand et al., 1990; Baliakina et al., 1999). Arvand et al demonstrated that α-toxin triggers a platelet signal that leads to secretion of intracellular contents including procoagulant mediators, platelet factor 4 and factor V. Secreted factor V in turn associates with the platelet membrane leading to assembly of the prothrombinase complex (Arvand et al., 1990). This explains the major pathway responsible for the procoagulatory effects of α-toxin. Bayer et al. used 2 models to investigate the role of α-toxin on platelets. In the first and consistent with the above observations, the authors demonstrated that α-toxin caused platelet lysis which in turn caused the release of platelet microbial proteins (PMP's). The release of PMPs from platelets was bactericidal to *S. aureus*. Using an animal model of endocarditis the authors demonstrated that different strains of *S. aureus* differed in the expression of functional versus mutant forms of α-toxin. Under these conditions, the *S. aureus* strains producing either minimal or no α-toxin were less virulent in vivo than wild-type strains (Bayer et al., 1997). Wild-type *S. aureus* strains or indeed an isogenic strain engineered to over-express α-toxin were associated with increased release of PMP from platelets. These results suggest that when *S. aureus* releases α-toxin in the vicinity of platelets it triggers them to release of PMP's and therefore forging a protective role for the host by destroying the α-toxin producing *S. aureus*.

Lipoteichoic acid (LTA) is a component of gram positive bacteria (Morath et al., 2005) and is often released from the bacteria upon lysis or after treatment with β-lactam antibiotics (Lotz et al., 2006). It also stimulates a strong immune response through an interaction with toll like receptors expressed on many host cells (Zahringer et al., 2008). Toll like receptor 2 (TLR2) recognises LTA and there are now many reports in the literature demonstrating that TLR2 is expressed and functional on platelets (Blair et al., 2009; Keane et al., 2010b; Kerrigan et al., 2008; Ward et al., 2005) suggesting that platelets can respond to LTA. Early reports demonstrated that *S. aureus* LTA inhibits platelet activation by activating the cyclic AMP pathway (Sheu et al., 2000a; Sheu et al., 2000b).

S. aureus predominantly uses multiple cell wall surface proteins to interact with platelets to trigger their activation. Early studies by Hawiger et al. demonstrated that *S. aureus* cell wall protein A acts as a receptor for specific anti-staphylococcal antibodies which in turn bind FcγRIIa on platelets (Hawiger et al., 1979). This interaction triggers an intracellular signal that leads to granule release and platelet aggregation. While this interaction is important, deletion of protein A failed to abolish *S. aureus* binding to platelets. This observation suggests that other interactions between *S. aureus* and platelets exist.

Clumping factor A (ClfA) is a 97kDa protein has been shown to bind plasma fibrinogen. A mutant of *S. aureus* lacking clumping factor A (ClfA) failed to adhere to platelets, suggesting that ClfA binds fibrinogen which in turn binds the platelet fibrinogen receptor, GPIIbIIIa (Sullam et al., 1996). Further characterisation of this interaction suggested that ClfA also requires IgG in order to trigger platelet aggregation. Addition of fibrinogen and ClfA-specific immunoglobulin to the plasma-free system led to *S. aureus*-induced platelet aggregation. Even though resting GPIIbIIIa has little or no affinity for soluble fibrinogen it can still bind fibrinogen bound to bacteria, however this is not enough to trigger activation. To trigger full platelet activation both fibrinogen and specific immunoglobulin must bind to the A domain on ClfA. There are two distinct sites on ClfA that allows fibrinogen and IgG binding at the same time (Loughman et al., 2005). Once bound fibrinogen molecules can engage resting GPIIbIIIa, aided by bound ClfA specific immunoglobulin, which encourages the clustering of Fc receptor, FcγRIIa. This triggers activation of signal transduction leading to conformational change in GPIIbIIIa and aggregation of platelets.

Deletion of the fibrinogen binding domain (ClfA-PY) but not the IgG binding domain on ClfA led to the discovery of a second pathway S. *aureus* uses to induce platelet aggregation. By removing the fibrinogen binding domain in ClfA *S. aureus* induced platelet aggregation very slowly (between 8-20 minutes compared to 2-4 mins). These results suggest that IgG binding to ClfA alone is not enough to trigger platelet aggregation. Using a series of elimination experiments Loughman et al. demonstrated that complement must assemble on the *S. aureus* surface and then bind to unidentified complement receptors on the platelet. Therefore, in the absence of fibrinogen binding complement and specific immunoglobulin are required for platelet activation to occur (Loughman et al., 2005).

A major limitation in our current understanding of platelet bacterial interactions stems from the fact that the majority of studies cited in the literature to date have been carried out under static conditions (static adhesion assays) or non-physiological stirring conditions (platelet aggregation). Therefore, data obtained using *in vitro* assays may not be relevant to the fluid shear environment that platelets encounter in the vasculature. Indeed many reports suggests that the local fluid environment of the circulation critically affects the molecular pathways of cell-cell interactions (Varki, 1994). All of the early *S. aureus* studies were carried out under static or non-physiological stirring conditions and therefore it is difficult to relate these studies to the disease processes. Studies using a cone and plate viscometer (a device that shears cells at a given flow rate) have demonstrated that protein A, ClfA, SdrC, SdrD and SdrE are important in thrombus formation (George et al., 2007; George et al., 2006; Pawar et al., 2004). However, extremely high shear rates were used in these rheological studies. Using another method of investigating the influence of shear rates on *S. aureus* ability to induce platelet activation, Kerrigan et al. perfused platelets over an immobilised monolayer of *S. aureus* in a parallel flow chamber. This method demonstrated that platelets perfused over *S. aureus* under shear conditions equivalent to arterial pressure led to very strong adhesion followed by rapid aggregate formation (Kerrigan et al., 2008). Deletion of ClfA from *S. aureus*, abolished adhesion and aggregate formation under all shear rates investigated. Using a plasma-free system, fibrinogen led to single platelet adhesion but not aggregate formation. Specific immunoglobulin failed to have any effect on either platelet adhesion or aggregation. However, addition of fibrinogen and specific immunoglobulin to the plasma-free system led to platelet adhesion followed by aggregate formation (Kerrigan et al., 2008) thus highlighting the importance of

fibrinogen and IgG in aggregate formation induced by *S. aureus*. No interaction was seen under low shear conditions using a parallel flow chamber.

S. aureus have a wide array of proteins expressed on their surface. This protein expression profile is most likely part of their survival. For example, ClfA is expressed weakly at the exponential phase of growth whereas is expressed strongly at the stationary phase of growth. In contrast to this another major *S. aureus* protein, fibronectin binding protein A (FnBPA) is strongly expressed at the exponential phase of growth and weakly expressed at the stationary phase of growth.

FnBPA is a 112kDa cell wall bound protein which binds plasma fibronectin and immunoglobulin. Fnbp contain a specific immunoglobulin binding domain (A domain) and a separate fibronectin binding domain (BCD). The FnBPA A domain is similar in structure and function to that of the ClfA A domain. FnBPA possesses two different but related mechanisms of engaging and activating platelets (Fitzgerald et al., 2006b). In the first mechanism, fibrinogen can bind to the A domain which crosslinks to GPIIb/IIIa, and specific immunoglobulin must crosslink to FcγRIIa to trigger platelet activation and aggregation (Fitzgerald et al., 2006b). In the second mechanism the fibronectin binding domain, BCD, can independently activate platelets. Fibronectin can bind to *S. aureus* via the FnBPA BCD domain by the tandem β-zipper mechanism (Meenan et al., 2007; Raibaud et al., 2005; Schwarz et al., 2003) and also to platelet GPIIbIIIa through the common integrin recognition motif RGD (Fitzgerald et al., 2006b). The signal to trigger platelet activation/aggregation is complete when specific immunoglobulin binds the A domain of FnBPA and cross links to platelet FcγRIIa.

Clumping factor B is a 98 kDa protein highly expressed on the surface of *S. aureus* during the exponential phase of growth and shares sequence homology with ClfA (McAleese et al., 2001). Similar to ClfA, ClfB can also bind fibrinogen and specific immunoglobulin. Deletion of the fibrinogen binding site on ClfB led to a slower aggregation. Characterization of this slower response suggested that complement assembly was required along with immunoglobulin to trigger aggregation (Miajlovic et al., 2007). Therefore similar to *S. aureus* ClfA and FnBPA, ClfB is also capable of triggering platelet aggregation via 2 specific mechanisms; fibrinogen and immunoglobulin or complement and immunoglobulin. Although the complement receptor on platelets has not yet been definitively identified in these interactions, Nguyen et al. has demonstrated that *S. aureus* protein A is capable of binding to the complement receptor gC1qR/p33 (Nguyen et al., 2000). As gC1qR/p33 is only expressed on platelets upon activation rather than at resting, it suggests that the complement interaction may act as an anchor, as it this interaction occurs after the initial platelet activation response.

Staphylococcal protein A (SpA) is a 55kDa protein expressed on greater than 90% of *S. aureus* strains. SpA is made up of 5 repeat domains (A-E) which have been shown to bind to the A1 domain of the major plasma protein vonWillebrand factor with high affinity (low nM range) (Hartleib et al., 2000; O'Seaghdha et al., 2006). Platelets express a high affinity vonWillebrand factor receptor called GPIbα (Andrews et al., 2003). Under very high shear conditions, Pawar and colleagues demonstrated that deletion of SpA from *S. aureus* significantly reduced its interaction with platelets (Pawar et al., 2004). Furthermore, preincubating platelet rich plasma with a vonWillebrand Factor antibody or indeed blocking the platelet GPIbα receptor with an inhibitory monoclonal antibody partially inhibited the

platelet-*S. aureus* interaction (Pawar et al., 2004). Thus highlighting the importance of this platelet specific receptor in recognising *S. aureus*.

Bacteria	Bacterial protein	Platelet receptor	Bridging protein	Response
Staphylococcus aureus	α-toxin	None	None	Aggregation
	Lipoteichoic acid	Not identified	None	Aggregation
	Protein A	FcγRIIa, gC1R/p33, GPIbα	IgG, complement, vonWillebrand factor	Aggregation / adhesion / thrombus formation
	ClfA	GPIIbIIIa, FcγRIIa,	Fibrinogen, IgG & complement	Aggregation / thrombus formation
	SdrC	Not identified	Not identified	Thrombus formation
	SdrD	Not identified	Not identified	Thrombus formation
	SdrE	Not identified	Not identified	Thrombus formation
	FnbpA/B	GPIIbIIIa, FcγRIIa	Fibrinogen, IgG & complement	Aggregation / thrombus formation
	ClfB	GPIIbIIIa, FcγRIIa	Fibrinogen, IgG & complement	
	SraP	Not identified	None	Thrombus formation
	IsdB	GPIIbIIIa	None	Aggregation / adhesion

Table 1. List of molecular interactions between Staphylococci and platelets that contribute to Infective Endocarditis

Serine rich protein SraP is a 227kDa large protein and a member of the highly conserved family of serine rich surface glycoproteins expressed on the cell wall of *S. aureus* (Siboo et al., 2005). A mutant strain of *S. aureus* lacking expression of SraP displayed signs of reduced virulence in a rabbit model of infective endocarditis, implicating its role in the development of a growing thrombus on a cardiac valve. The platelet receptor that SraP binds to is not currently known. SraP shares similarility with a group of cell wall-associated glycoproteins (Ramboarina et al., 2010) found in a number of other organisms including *Streptococcus sanguinis* (Plummer et al., 2005), *Streptococcus gordonii* (Kerrigan et al., 2007) both of which have been have been shown to bind to platelet GPIbα. Interestingly however, SraP does not seem to bind to GPIbα.

In vivo, *S. aureus* has restricted access to iron and as a result express iron regulated surface proteins to capture haem from haemogloblin and transport it into the cell (Skaar and Schneewind, 2004). Iron-regulated surface determinant B (IsdB) is a 70kDa family member that has been shown to bind to the platelet fibrinogen receptor GPIIbIIIa. *S. aureus* grown in iron limited conditions bound to platelets in a plasma free environment, suggesting that a plasma bridge bridge is not necessary for interacting with or inducing platelet activation. Mutants defective in the expression of IsdB were unable to adhere to or aggregate platelets. Using surface plasmon resonance Miajlovic et al demonstrated a direct interaction between purified GPIIbIIIa and recombinant IsdB (Miajlovic et al., 2010).

In addition to *S. aureus* having the ability to interact with platelet either directly or indirectly Youssefian and colleagues reported that platelets were also capable of internalising *S. aureus* (Youssefian et al., 2002). While internalisation corresponded with platelet activation the mechanism through which this occurred is currently not known. Moreover it is also yet to be established whether the *S. aureus* is destroyed once internalised in a manner similar to phagocytosis by immune cells.

7. Platelet-bacterial interactions: The Streptococcus

Viridans streptococci are common commensals of the oral cavity, respiratory tract, and gastrointestinal mucosa. In their respective environment these microorganisms are harmless, however following trauma to the mucous membranes they can enter to the normally sterile environment of the bloodstream. Once inside the bloodstream the viridans streptococci act as pathogens (Baddour et al., 1989) and often colonise heart valves, causing infective endocarditis (Moreillon et al., 2002) or become implanted in atherosclerotic plaques, exacerbating atherosclerosis (Chiu, 1999) mainly through an interaction with platelets (Table 2).

Streptococcus sanguinis is a common oral microorganism that is isolated from patients with infective endocarditis. In fact early studies demonstrated that 60% of *S. sanguinis* strains induced platelet aggregation in vitro (Herzberg and Meyer, 1996). Douglas et al provided strong evidence that there is a relationship between the virulence of the infecting *S. sanguinis* strains and their ability to aggregate platelets *in vitro (Douglas et al., 1990). S. sanguinis* induced platelet aggregation was largely found to be dependent on physiological concentrations of calcium and fibrinogen, and some strains required non-specific antibody. Platelet adhesion to *S. sanguinis* occurred independently of calcium, fibrinogen or non-specific antibody, suggesting that platelet adhesion and platelet aggregation are mediated independently of each other (Kerrigan et al., 2002). These observations led to the early model of platelet bacterial interaction where a class I component mediated adhesion to the platelet, a class II component mediates a calcium dependent activation of the platelet and finally a class III component mediates a calcium and fibrinogen-dependent amplification of the response (Herzberg, 1996).

The initial studies focused on identifying the class I and class II components. Treatment of *S. sanguinis* with L- (tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-trypsin liberated cell free peptides (Herzberg et al., 1983). Purification of these peptides failed to support platelet adhesion or trigger platelet aggregation, however, did inhibit *S. sanguinis* mediated platelet aggregation and adhesion. This studies concluded that that these TPCK-tryptic peptides contained antigenic determinants that recognise platelets thus preventing *S.*

sanguinis from binding. Using immunoaffinity chromatography and ion exchange chromatography, Erickson and colleagues identified a platelet aggregation-associated protein (PAAP) (Erickson and Herzberg, 1990). PAAP is synthesized as a 150 kDa glycoprotein which is 40% carbohydrate and is constitutively expressed on the surface of *S. sanguinis* (Erickson and Herzberg, 1993; Erickson et al., 1992). The peptide sequence pro-gly-glu-gln-gly-pro-lys in the PAAP conforms to a predicted consensus motif common to the platelet interactive domain of collagen, lys-pro-gly-glu-pro-gly-pro-lys (Erickson and Herzberg, 1990). More recently Herzberg and colleagues identified a putative collagen-binding protein containing 2 PAAP sequences (Herzberg et al., 2005). PAAP is environmentally controlled during infection in response to high temperature (fever) or exposed collagen (exposed on damaged blood vessels or damaged heart valves) (Heimdahl et al., 1990). Partial sequence alignment shows homology of PAAP to heat shock proteins of *Mycobacterium tuberculosis* and *E. coli* (Herzberg and Meyer, 1996). The platelet receptor for PAAP is currently unidentified.

Ford et al demonstrated that *S. sanguinis* strain NCTC 7863 induced aggregation of normal platelets suspended in plasma however removal of plasma proteins abolished platelet aggregation (Ford et al., 1996). The long lag time (12-15mins) of *S. sanguinis* strain 7863 was progressively shortened by incubating the bacteria in plasma for increasing lengths of time prior to addition to platelets. This suggested that plasma proteins were essential for platelet aggregation. Subsequent experiments demonstrated that complement assembly on the surface of the bacteria was necessary for aggregation of platelets. Further experiments demonstrated that complement assembly was not enough to trigger platelet aggregation which led to the discovery that IgG and fibrinogen was necessary to complete the aggregation process (Ford et al., 1997). More recently McNicol and colleagues demonstrated that depletion of *S. sanguinis*-specific antibodies from plasma prevented platelet aggregation (McNicol et al., 2006). Following *S. sanguinis* binding, addition of antibodies led to rapid phosphorylation of the platelet antibody receptor, FcγRIIa (Pampolina and McNicol, 2005) and further downstream effector targets such as phospholipase Cγ2, syk and adapter molecule LAT.

In 2002 Kerrigan et al, demonstrated that *S. sanguinis* strain 133-79 bound to the platelet vonWillebrand factor receptor, GPIbα (Kerrigan et al., 2002). There was no requirement for vonWillebrand factor or immunoglobulin binding in this system, suggesting that *S. sanguinis* binds directly to platelet GPIbα. Confirmation for the role of GPIbα was provided when a range of site-specific inhibitory monoclonal antibodies against GPIbα prevented *S. sanguis* binding to the platelet. In addition, enzymatic cleavage of GPIbα using the snake venom from a viper pit localised the binding region within the N-terminal 1-225 portion of GPIbα. Finally, platelets from patients with Bernard Soulier Syndrome (patients who fail to express GPIbα on the surface of their platelets) did not aggregate in response to *S. sanguinis* (Kerrigan et al., 2002). The *S. sanguinis* protein that binds GPIbα was purified from cell wall extracts by chromatography on GPIbα and wheat germ agglutinin affinity matrices (Plummer et al., 2005). This led to the identification of a highly glycosylated serine-rich protein called serine-rich protein A (SrpA). An insertional inactivation mutant lacking the SrpA of *S. sanguinis* showed a significant increase in the lag time to aggregation, implicating this protein in platelet aggregation. In addition, platelet adhesion to the SrpA mutant was significantly reduced, however not abolished, suggesting other factors are involved in supporting platelet adhesion.

Streptococcus gordonii is a close but distinct relative of *S. sanguinis* (Nobbs et al., 2009), however initial reports suggested that *S. gordonii* could not induce platelet aggregation (Douglas et al., 1990). More in depth characterisation has demonstrated that *S. gordonii* is capable of supporting platelet adhesion and inducing platelets aggregation in a strain dependent manner. Glycosylated surface protein B is a large 280kDa protein expressed on the surface of *S. gordonii* strain M99. It is heavily glycosylated with glucose and glucosamine and is transported to the cell surface via an accessory system compromising of the SecA2 and the SecY2 proteins (Bensing and Sullam, 2002; Takahashi et al., 2004). GspB has been shown to be highly homologous to an expanding family of Gram-positive bacterial cell surface proteins that includes *S. aureus* serine rich protein SraP (Siboo et al., 2005) *S. sanguis* serine-rich protein SrpA (Plummer et al., 2005), *S. gordonii* DL1 sialic acid-binding protein Hsa (Takahashi et al., 2002) and the *S. parasanguinis* fimbriae-associated protein Fap1 (Wu et al., 2007). These proteins were originally discovered because of their ability to bind a variety of sialylated glycoproteins. Platelet GPIbα is an example of a sialylated glycoprotein and as a result has been shown to bind *S. gordonii* GspB and Hsa (Bensing et al., 2004). Additional molecular glycan characterisation demonstrated that GspB specifically binds O-linked sialic acid residues on GPIbα (Takamatsu et al., 2005), whereas Hsa specifically binds N-linked sialic acid residues on GPIbα and GPIIbIIIa (Yajima et al., 2005). Interestingly an isogenic mutant lacking the expression of Hsa in *S. gordonii* strain DL1 failed to affect percent platelet aggregation however reduced platelet adhesion by ~ 50%. These more recent functional studies suggest that additional protein interactions are necessary for supporting platelet adhesion (Jakubovics et al., 2005a).

S. gordonii expresses a large protein of ~3500 amino acids with a high molecular weight of 397 kDa designated platelet adherence protein A (PadA) (Petersen et al., 2010). PadA contains a short stretch of amino acid residues which displays weak homology to A1 and C1 domain of the plasma protein, vonWillebrand factor (vWf). These domains in vWf are essential for interactions with platelet GPIb and GPIIb/IIIa, respectively. Disruption of the PadA gene from *S. gordonii* DL1 failed to affect binding to glycocalacin (soluble purified GPIbα), however, ablated binding to purified GPIIb/IIIa. Furthermore, platelet adhesion to *S. gordonii* DL1 was significantly reduced by preincubation of platelets with an integrin recognition RGD-containing peptide or the GPIIb/IIIa inhibitor, abciximab (Petersen et al., 2010). Earlier studies demonstrated that *S. gordonii* DL1 bound specifically to GPIIb but not GPIIIa of the GPIIbIIIa complex (Yajima et al., 2005). Collectively these results suggest that platelet adhesion is a mutlifactorial event where Hsa binds GPIbα and PadA binds GPIIb/IIIa and together they act synergistically to support platelet adhesion.

Platelets are very sensitive to shear and some platelet-substrate interactions only manifest themselves upon exposure to shear. For example, under low shear conditions there is no interaction between platelets and vWf however under high shear conditions platelets roll along the vWf coated surface (Ruggeri, 2009). Perfusing platelets over immobilised *S. sanguinis* or *S. gordonii* in a parallel flow chamber under low shear (veneous shear), platelets interacted with a typical rolling behaviour followed by firm adhesion (Jakubovics et al., 2005a; Plummer et al., 2005). This phenomena is highly characteristic of platelet rolling on damaged endothelium (Ruggeri, 2009). Rolling is mediated by platelet GPIbα where is the fast on-off rate of the receptor allows loss of interaction between GPIbα and the bacteria on one side of the platelet leading to the formation of another GPIbα-bacterial interaction on

the other side of the platelet. Deletion of the *S. sanguinis* GPIbα-binding protein SrpA or indeed the *S. gordonii* GPIbα-binding protein Hsa abolished the rolling behaviour, suggesting that these proteins form the initial attachment of platelets with the bacteria under physiological shear conditions. Soluble vWf exists in the plasma in a conformation that is not recognisable by platelet GPIbα. However at the site of injury, vWf binds to extracellular matrix proteins forming a thrombotic surface for the platelet. Typically platelets roll along immobilised vWf under high shear conditions. The high shear induces a conformational change in vWf making it recognisable to GPIbα. As *S. gordonii* Hsa and *S. sanguis* SrpA can support static platelet adhesion, or indeed mediate rolling of platelets under low shear conditions it suggests that Hsa and SrpA exist in a unique conformation that is recognisable by GPIbα, as their receptor conformation is not believed to be altered when subjected to shear. The process of rolling is to slow the platelet down from the high shear force experienced in the vasculature long enough to allow it firmly adhere to the bacteria. Firm adhesion is complete when platelet GPIIb/IIIa interacts with *S. gordonii* PadA.

Once the platelets become firmly adhered to *S. gordonii* by engaging with either GPIbα or GPIIbIIIa, a signal is generated in the platelet that results in the platelet changing shape and spreading out on the bacterial surface. The function of platelet spreading is essential for the platelet to withstand the shear forces experienced in the vasculature. Platelet spreading is a particularly important in the development of thrombotic vegetations in infective endocarditis because the lesions around the lesion on the cardiac valve are often very turbulent. Therefore, platelets require the conversion from a discoid shape to a fully spread cell to withstand turbulent shear force. Keane et al, demonstrated that engagement of either GPIbα or GPIIbIIIa, the ITAM-bearing receptor, FcγRIIa and its downstream effectors syk and phospholipase Cγ2 became tyrosine phosphorylated which suggests that this pathway is essential for platelet spreading (Keane et al., 2010a). In addition tyrosine phosphorylation of the FcγRIIa resulted in platelet degranulation, a step critical for inducing and amplyfing platelet aggregation.

Once platelets become firmly adhered and a signal is generated in the platelet it leads to platelet aggregation. Interestingly, while a mutant *S. gordonii* strain lacking expression of Hsa displayed reduced ability to support platelet adhesion, platelet aggregation remained unaffected. This observation suggests that different proteins expressed on the surface of *S. gordonii* mediate different interactions and subsequent responses in platelets. To address this Kerrigan et al used a proteomic approach to identify differentially expressed proteins on a strain of *S. gordonii* that induces platelet aggregation (*S. gordonii* strain DL1) versus one that does not (*S. gordonii* strain Blackburn). Cell wall proteins from both strains were removed from the cell wall using lysozyme. Recovered proteins were separated by poly-acrylamide gel electrophoresis and 2 bands corresponding to 172 kDa and 164 kDa were shown to be differentially expressed. Mass spectrometry identified these proteins as antigen I/II (Kerrigan et al., 2007). The antigen I/II family of proteins are probably the best characterised proteins on the surface of *S. gordonii*. Originally identified on *Streptococcus mutans*, antigen I/II have now been identified on almost all oral streptococci (Nobbs et al., 2007). In *S. gordonii*, antigen I/II have been designated SspA/B. These proteins are oligospecific adhesins which have been shown to bind to several ligands such as collagen type I (Heddle et al., 2003), β$_1$ integrins (Nobbs et al., 2007), salivary agglutinin glycoprotein (Prakobphol et al., 2000) as well as other bacteria including *P. gingivalis*, *Candida albicans* and *Actinomyces*

naeslundii (Demuth et al., 2001; Egland et al., 2001; Jakubovics et al., 2005b). Deletion of SspA/B from *S. gordonii* DL1 failed to affect either platelet aggregation or platelet adhesion. However, deletion of SspA/B and Hsa together abolished platelet aggregation and reduced platelet adhesion by 50%, similar to the Hsa mutant alone (Kerrigan et al., 2007). Consistent with this over expression of SspA and SspB in the non-platelet reactive surrogate host *Lactococcus lactis* induced platelet aggregation but failed to support platelet adhesion. These results suggest that SspA/B and Hsa must act synergistically when binding to platelets to trigger aggregation and that they are not involved in supporting platelet adhesion. At present the receptor through which SspA/B binds to on the platelets is not current known.

Bacteria	Bacterial protein	Platelet receptor	Bridging protein	Response
S. sanguinis	PAAP	Not identified	None	Aggregation
	Not identified	FcγRIIa	Antibody, fibrinogen & complement	Aggregation
	SrpA	GPIbα	None	Adhesion / aggregation
S. gordonii	GspB	GPIBα	None	Adhesion
	Hsa	GPIbα	None	Adhesion / aggregation
	PadA	GPIIbIIIa	None	Adhesion
	SspA/B	Not identified	None	Adhesion / Aggregation
S. mitis	PblA/B	Not identified	None	Aggregation
	Lysin	GPIIbIIIa	fibrinogen	Aggregation
S. mutans	Rgp	Not identified	IgG	Aggregation
	PAc	Not identified	None	Aggregation
S. oralis	Not identified	Not identified	None	Adhesion / aggregation
S. parasanguinis	FimA	Not identified	None	?
	Fap1	Not identified	None	Adhesion
S. pneumoniae	Not identified	TLR2	None	Aggregation

Table 2. List of molecular interactions between Streptococci and platelets that contribute to Infective Endocarditis

Streptococcus mitis has also been shown to interact with platelets although reports are conflicting. In 1990, Douglas et al, used several strains of *S. mitis* and found that none of these interacted with platelets. Ohkuni and colleagues suggested that *S. mitis* strain Nm-65

released a 66 kDa protein (*S.mitis*-derived human platelet aggregation factor, Sm-PAF) that induced platelet aggregation (Ohkuni et al., 1997). Characterisation of this secreted protein revealed that it was a toxin that lysed platelets rather than induced platelet aggregation. More recent work by Bensing et al, identified two distinct genetic loci of *S. mitis* strain SF100 that contributes to platelet binding (Bensing et al., 2001a). The first locus encodes PblT. The mechanism through which it binds to platelets has not yet been explored. The second locus encodes two cell wall associated proteins; PblA is a 107 kDa protein and PblB which is a 121 kDa protein (Bensing et al., 2001b). These proteins are unique as neither of the proteins expresses homology to any other bacterial adhesins described, however share similarities with structural components of bacteriophages (Mitchell et al., 2007). Bacteria often bind to oligosaccharides on host cells. For example *S. gordonii* GspB binds to α2-3-linked sialic acids on platelets and salivary mucins. Platelets treated with sialidases having different linkage specificities demonstrated that removal of α2-8-linked sialic acids resulted in a reduction in *S. mitis* SF100 binding. Gangliosides are glycosphingolipids rich in sialic acids and are found in abundance in lipid rafts in platelets (Marcus et al., 1972). *S. mitis* strains lacking the expression of PblA and PblB demonstrated significant decrease in platelet binding invitro as well as marked reduction in virulence in an animal model of infective endocarditis (Bensing et al., 2001b; Mitchell et al., 2007). More recent studies have demonstrated that *S. mitis* surface bound lysin can bind both free and platelet bound fibrinogen, through its interaction with the Aa and Bb chains of fibrinogen (Seo et al., 2010). Once *S. mitis* has bound fibrinogen this in turn can bind to the platelet fibrinogen receptor, GPIIbIIIa, initiating thrombus formation.

Although early reports suggest *Streptococcus oralis* is capable of inducing platelet aggregation and supporting platelet adhesion the exact mechanism through which either of these occur is currently unknown.

Streptococcus mutans strain Xc induces platelet aggregation by releasing extracts containing serotype-specific polysaccharides which are composed of rhamnose-glucose polymers. An isogenic mutant lacking synthesis the rhamnose-glucose polymers significantly reduced platelet aggregation (Chia et al., 2004). *S. mutans* failed to induce platelet aggregation in the absence of plasma proteins suggesting that a plasma protein possibly bridges the bacteria to the platelet. Subsequent studies identified that addition of serotype-specific IgG to plasma free platelets restored aggregation. The ability of rhamnose polymers toinduce platelet aggregation has been known for some time. For example, ristocetin is obtained from Amycolatopsis lurida and is well characterised for its ability to induce platelet agglutination. The mechanism of ristocetin induced agglutination of platelets involves vWf binding to platelet GPIbα. Cleavage of the rhamnose tetrasaccharide of ristocetin abolished its ability to induce platelet aggregation (Bardsley et al., 1998). Moreover, *S. sanguinis* expresses a platelet associated aggregating protein (PAAP) which is another rhamnose polymer which is thought to be involved in inducing platelet aggregation. Together these results suggest that rhamnose plays an important role in inducing platelet aggregation. Additional studies by Munro et al, demonstrated that an isogenic mutant of *S. mutans* strain V403 lacking the exopolysaccharides glucan and fructan had decreased infectivity in the rat model of endocarditis compared to the wild-type strain (Munro and Macrina, 1993). Protein antigen C (PAc) is a high molecular weight protein expressed by *S. mutans* that has been shown to interact with an unidentified platelet protein (Matsumoto-Nakano et al., 2009). Clinical strains that do not naturally express PAc failed to induce platelet aggregation.

Although the platelet receptor that recognises PAc was not identified, the authors demonstrated that increasing amounts of anti-PAc serum significantly reduced platelet aggregation, suggesting that antibody recognition may be a critical factor.

Streptococcus parasanguinis expresses a 36kDa surface protein called FimA. A mutant defective on the expression of FimA from *S. parasanguinis* strain FW213 failed to have any effect on supporting platelet adhesion or inducing platelet aggregation however significantly reduced the extent of endocarditis in the rat model (Burnette-Curley et al., 1995). Based on these observations the exact mechanism by which FimA functions as a virulence factor of endocarditis has yet to be determined, however it is possible that FimA plays a role in adherence to fibrin deposits associated with damaged heart valves. Fimbrae-associated protein 1 (Fap1) is a highly glycosylated serine-rich glycoprotein which shares sequence and structural homology with SrpA of *S. sanguinis*, GspB of *S. gordonii* and SraP of *S. aureus*. SrpA of *S. sanguinis* and GspB of *S. gordonii* have both been shown to interact directly with platelet GPIbα to trigger platelet activation (Wu et al., 2007). To date no studies have demonstrated that Fap1 interacts with GPIbα or indeed with platelets directly.

Early studies by Johnson et al, demonstrated that at low concentrations *Streptococcus pneumoniae* pneumolysin had little effect on platelets, however at higher concentrations platelets were lysed presumably by binding to the sterols present in the platelet plasma membrane and forming multimeric transmembrane pores (Johnson et al., 1981). The ability to form these pores may result in lysis of platelets. Lysis occurred in a time-dependent manner where greater than 50% of platelets were lysed. A more recent study demonstrated that a concentrated sample of supernatant from an overnight growth of *S. pneumoniae* that contained pneumolysin failed to induce human platelet aggregation, moreover a pneumolysin-negative strain of *S. pneumoniae* also induced platelet aggregation suggesting that pneumolysin is not involved in inducing platelet aggregation (Keane et al., 2010b). In this report the authors demonstrated that *S. pneumoniae* binds to platelet toll like receptor 2 (TLR2), a pattern recognition receptor which triggered the PI3kinase/RAP1 pathway which leads to activation of the fibrinogen receptor GPIIbIIIa.

8. Platelet-bacterial interactions: Non staphylococcal, non streptococcal

There is no doubt Staphylococcus and Streptococcus species make up the majority of cases of infective endocarditis, however other opportunistic pathogens, while rare, may also play a role (Table 3). For example recent work by Shannon et al. demonstrated that clinical isolates of *Enterococcus faecalis* induced platelet aggregation. The ability of the *E. faecalis* to induce platelet aggregation was dependent on IgG (Rasmussen et al., 2010). *Helicobacter pylori* has also been shown to interact with platelets. When in plasma it binds vonWillebrand factor, which in turn enables it to interact with GPIbα and trigger platelet aggregation. This is a unique interaction as soluble vonWillebrand factor cannot interact with platelet GPIbα. The *H. pylori* protein that binds vonWillebrand factor is currently not identified (Byrne et al., 2003; Corcoran et al., 2007). *Porphyromonas ginigvalis* secretes a protease called gingipains that acts directly on platelet protease activated receptors (Lourbakos et al., 2001). Naito and colleagues also deomstrated that *P. ginigvalis* expresses a protein called Hgp44 which also induces platelet aggregation. Although the platelet receptor has not been definitively identified antibodies against GPIbα abolish the interaction (Naito et al., 2006). Finally, *Escherichia coli* secretes shiga-like toxin which binds

to the glycosphingolipid receptors on the surface of the platelet and triggers platelet activation (Cooling et al., 1998), however other groups failed to see the interaction in vivo (Viisoreanu et al., 2000). One explanation for these contradictory results is that in vivo the actions of Shiga toxins are complex and many of its actions on platelets are indirect being mediated through effects on other cells such as monocytes (Guessous et al., 2005) and endothelial cells (Motto et al., 2005).

Bacteria	Bacterial protein	Platelet receptor	Bridging protein	Response
Enterococcus faecalis	Not identified	FcgRIIa	IgG	Aggregation
Helicobacter pylori	Not identified	GPIbα	vonWillebrand Factor	Aggregation
Porphyromonas gingivalis	RgpA/B	PAR1 & PAR4	None	Aggregation
	Hgp44	GPIbα	Not identified	Aggregation
Escherichia coli	Shiga-like toxin	Sphingolipids	None	Aggregation

Table 3. List of molecular interactions between non staphylococcal non streptococcal microorganisms and platelets that may contribute to Infective Endocarditis

9. Current and novel treatment options for infective endocarditis

As infective endocarditis is primarily a bacteria infection, often associated with dental and other medical procedures, prophylactic antibiotic treatment was considered to be normal practice. However, more recently the American Heart Association (AHA) and American College of Cardiology (ACC) released a guidance document on infective endocarditis that significantly changed routine practice (Nishimura et al., 2008). In particular they no longer recommend the prophylactic use of antibiotics for dental or other procedures except for a small group of high-risk patients. This was primarily due to the fact that the risks associated with prophylaxis outweigh the benefits. The review panel felt that the biggest risk for IE is not dental or other procedures but rather bleeding from gums due to poor oral hygiene. Thus, so few cases of IE will be prevented by antibiotic prophylaxis during medical procedures but there will be an increase in antibiotic resistance that the there is little benefit to prophylaxis. They felt that improved oral hygiene would be more effective at preventing the occurrence of IE. The exception was for very high-risk patients, which are basically patients with prosthetic valves or those with a history of IE.

With the increase in antibiotic-resistant strains of pathogenic bacteria researchers have been looking at new strategies to target bacteria. One such approach is to target the host response to the pathogen (Clatworthy et al., 2007). IE is very amenable to this strategy as it basically arises from an ineffective host response to the infection. If the platelet aggregation response can be prevented there would be no thrombus formation.

The obvious strategy would be to use an anti-platelet agent to prevent the thrombus formation. Aspirin is the most widely used anti-platelet agent and acts to inhibit cyclooxygenase but may not be effective in preventing IE (Chan et al., 2003). While aspirin effectively inhibits COX some bacteria can activate platelets in a COX-independent manner. As it is not known prior to the procedure which bacteria are likely to be involved aspirin would not be sufficient. A more effective strategy would be to use a $P2Y_{12}$ antagonist such as clopidogrel. However, its very effectiveness makes it unlikely to be used. The inhibition of platelet function by clopidogrel and other anti-platelet agents cause bleeding and it is unlikely that dentists would be happy to perform extractions and other such procedures when the patient is likely to experience very heavy bleeding. Certainly surgeons will not operate on a patient on clopidogrel and they typically require a reversal of the inhibition before they will consider surgery.

Targeting the bacteria is an approach that is attractive, as it would not interfere with platelet function. However, as we have seen choosing a target is difficult as every species of bacteria interact differently with platelets and even different strains of the same species can interact differently (Fitzgerald et al., 2006a). There is also the problem that bacteria such as *S. aureus* have multiple interactions with platelets and it is not clear which one if any is the critical one during IE. Certainly if it was possible to identify a single bacterial protein that was conserved across many pathogenic strains and was found to be critical in the interaction with platelets it would be possible to produce a vaccine against it (Broughan et al., 2011).

Despite the limitations associated with the use of anti-platelet agents platelets are still a promising target for any potential new drug. This is because so far there are only a few platelet receptors implicated in the response to bacteria primarily GPIIb/IIIa and GPIbα. GPIIb/IIIa plays an important role in *S. aureus*-and *S. gordonii* mediated platelet aggregation but as the fibrinogen receptor it also plays a critical role in platelet aggregation. Because of this important role in platelet function a number of inhibitors of this receptor have been developed. Although only for intravenous use these drugs have been very effective anti-platelet agents (Curtin, 2004). However, they are very potent in their inhibition and will cause a profound inhibition of platelet function along with a prolongation of bleeding time that will not be suitable for prophylaxis during procedures such as dental manipulations.

A second platelet receptor that is important in the platelet response to bacteria is GPIbα, which is the von Willebrand factor receptor. GPIbα is very important in platelet adhesion under shear stress and is a very attractive target in cardiovascular disease. However, despite the efforts of many researchers to discover GPIbα inhibitors none have yet made it to the market (Vanhoorelbeke et al., 2007).

FcγRIIa has been shown to mediate platelet activation by many different bacteria including Staphylococci and Streptococci. It typically acts in conjunction with GPIbα, GPIIb/IIIa or the complement receptor. Unlike receptors such as GPIbα and GPIIb/IIIa FcγRIIa is not involved in thrombosis as its role are primarily involved in the immune function of platelets. Thus, antagonists of FcγRIIa will have little impact on platelet function and will not cause bleeding problems making it an ideal target for prophylactic therapy for IE.

The interaction between platelets and bacteria is the key element of infective endocarditis. While there are many different bacterial proteins that can bind to platelets there are only a few potential receptors on the platelet for these proteins. This makes the platelet a good

target for novel strategies to inhibit this interaction. While there is always a risk of causing bleeding problems with anti-platelet agents FcγRIIa has been found to be the critical receptor on platelets for the interaction with bacteria. As this receptor is associated with the innate immune functions of platelets rather than with its role in thrombosis targeting FcγRIIa will have minimal effects on the role of platelets in thrombosis. This strategy would be in line with the recent guidance document on infective endocarditis from AHA/ACC. Prophylactic use of an FcγRIIa antagonist during medical procedures with no risk of developing resistance. Antibiotic therapy could then be reserved for cases of sepsis or confirmed infective endocarditis.

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