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# Platelet Interactions with Bacteria

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## 1. Introduction

It is well established that the primary function of platelets is their adhesion to endothelium or to matrix protein components at sites of the injured vessel wall in the initiation of haemostasis [1]. Despite this critical role, platelets are poorly appreciated for their involvement in inflammatory or immune processes associated with host defence. The concept that platelets interact with bacteria is not new as there are many reports published throughout history describing this interaction. For example, the earliest report by Levaditi in 1901 demonstrated that platelets activated and 'clumped' when *Vibrio cholerae* were introduced into the circulation of rabbits [2]. In 1931 Dudgeon demonstrated platelet clumping occurred at 5 minutes of injecting *S. aureus* into rabbits [3]. More recently in the early 1970's Clawson and White demonstrated that bacteria were able to binding specifically to a platelet receptor, triggering a signal that resulted in aggregation and degranulation of intracellular contents [4-7]. These early studies provided observational evidence that platelets can respond to invading pathogens.

As our knowledge of basic platelet biology developed in more modern times it has become apparent that platelets are powerful multifunctional cells that are involved in processes outside their traditional role of thrombosis and haemostasis. For example, platelets share many similarities with professional leukocytes (white blood cells) well characterised for their role in immuno-protection following invasion by foreign invaders. Platelets can also recognise foreign invaders through specific receptors, release their granule contents and recruit immune cells.

Recently human platelets have been shown to express Toll Like Receptor (TLR) 1, 2, 4, 6, 8 and 9 [8-13]. These type I integral membrane receptors recognise common pathogen-associated molecular patterns found in foreign invaders. Platelets also express Intracellular Cell Adhesion Molecular (ICAM) 2 which binds to leukocyte  $\beta 2$  integrin, LFA-1 ( $\alpha 1\beta 2$ , CD11a/CD18) and to

dendritic cell specific ICAM grabbing nonintegrin (DC-SIGN). Trans interactions of platelet-derived Junctional Adhesion Molecules (A and C) have been found to support the luminal deposition of platelet chemokines and to enhance the recruitment of leukocytes. Upon activation CD40L is upregulated on the platelet surface which results in stimulation of endothelial cells through its cognate receptor CD40 and in increased expression of adhesion molecules, release of chemokines (eg. RANTES) enhancing recruitment of leukocytes [14].

As a result of such receptor mediated interactions platelets can secrete granular contents which have significant immuno-modulatory effects. Alpha granules contain proteins such as P-selectin which mediates adhesion of platelets to monocytes, neutrophils and lymphocytes, resulting in the formation of platelet leukocyte complexes [15-17]. Secretion also results in release of many chemotactic agents which lead to the recruitment of various inflammatory cells; platelet derived growth factor (PDGF) and 12-hydroxyeicosatetraenoic acid (12-HETE) which recruit neutrophils [18, 19]; platelet factor 4 and platelet derived histamine releasing factor (PDHRF) which recruit eosinophils in airway disease [20, 21]; PDGF and transforming growth factor  $\beta$  (TGF- $\beta$ ) which recruit monocytes and macrophages and TGF- $\beta$  which recruits fibroblasts [22-24]. In addition the alpha granules also release many antimicrobial peptides such as beta-lysin, platelet microbial protein (PMP), neutrophil activating peptide (NAP-2), released upon activation normal T-cell expressed and secreted (RANTES) and fibrinopeptides A and B [25-29].

## 2. Common observations in platelet-bacterial interactions

Unlike typical platelet agonists that bind to specific platelet receptors and trigger a response, bacteria can interact with platelets using a number of different mechanisms.

*Direct interaction:* Bacteria express proteins that can directly interact with a surface receptor on the platelet. In this case they have ligand-mimetic domains that act as agonists on the platelet receptor. One such direct interaction is that with *Streptococcus sanguinis* which can directly to GPIIb $\alpha$ , the vWf receptor on the platelet [30]. Other potential mediators of platelet activation are lipopolysaccharide (LPS) and lipoteichoic acid (LTA). LPS has been shown to activate platelets and LTA has been shown to inhibit platelet activation [11, 31].

*Indirect interaction (bridging protein):* Bacteria can also coat itself in a plasma protein and then use this mechanism as a bridge to its reciprocal platelet receptor. For example, Clumping factor A & B [32] and fibronectin binding protein [33] on *S. aureus* can both bind fibronectin and/or fibrinogen both of which are ligands for GPIIb/IIIa on the platelet. *Helicobacter pylori* can bind vWf which interacts with GPIIb $\alpha$  on the platelet [34]. The most common bridging molecule for bacteria to use is IgG. IgG bound to the bacteria surface can interact with the platelet Fc $\gamma$ RIIa receptor and while it appears that this alone cannot stimulate platelet activation it acts in conjunction with other bridging molecules [34-36]. In the absence of a second bridging molecule bound antibody can trigger complement formation which can mediate platelet activation via complement receptors in conjunction with the Fc $\gamma$ RIIa receptor [36-38].

*Indirect interaction (secretion)*: Bacteria also have the potential to secrete products that can in turn activate platelets. *Porphyromonas gingivalis* secretes gingipain, an enzyme that activates the thrombin receptor on platelets which leads to platelet activation [39] and *Escherichia coli* shiga toxin is associated with platelet activation [40] via a novel platelet glycosphingolipid [41]

Bacterial induced platelet aggregation is different in some respects to that seen with other platelet agonists. Bacterial-induced aggregation is an all-or-nothing response, in that no matter what concentration of bacteria are added to a platelet preparation the extent of aggregation will always be maximal (often less than that seen with other agonists) or else there is no aggregation. Unlike other agonists there is a lag time to aggregation. Adjusting the concentration of bacteria shortens the lag time to a minimum but never eliminates it. There are two categories of bacteria: those that have a short lag time of around 2-5 mins e.g. *S. aureus* and those with a long lag time of 15-20 mins e.g. *S. sanguinis* or *S. gordonii*. The short lag time usually indicates the presence of a direct interaction and is dependent on the levels of expression of the interacting protein on the bacterial surface [36]. The long lag time usually indicates a complement –dependent aggregation process.

Bacteria can finally support platelet adhesion, induce platelet spreading or trigger platelet aggregation and these interactions are often mediated by different platelet receptors and bacterial proteins. For example, *S. gordonii* supports platelet adhesion via a GPIb $\alpha$  – Hsa/GspB interaction, whereas platelet spreading is mediated by GPIIb/IIIa –PadA interaction and finally platelet aggregation is mediated by GPIb $\alpha$  – Hsa/GspB/SspA/B interaction.

### 3. Platelet receptors recognised by bacteria

#### 3.1. Glycoprotein Ib $\alpha$

##### 3.1.1. Direct interaction with GPIb $\alpha$

*Streptococcus sanguinis* and *Streptococcus gordonii* are common commensals found in the oral cavity and have been found to bind directly to GPIb $\alpha$ . The interaction is mediated by a growing family of bacteria adhesins called Serine Rich Repeat (SRR) proteins. *Streptococcus sanguinis* expresses SrpA and *Streptococcus gordonii* expresses GspB and Hsa. [30, 42, 43]. The region on GspB and Hsa that bind to GPIb $\alpha$  has been localised to the non-repetitive region and interestingly does not contain any sequence similarity with vWf [44]. The non-repetitive ligand binding region of GspB has been worked out by crystallography studies as having a modular organization: helical domain; a Siglec domain (domain that binds sialic acids); and a unique domain [45]. A mammalian carbohydrate binding domain, identified as the Siglec domain, was found in Hsa and SrpA but not in the protein sequences of five other characterised SRR proteins on other bacteria suggesting that this domain is critical for the interaction with GPIb $\alpha$  [45]. Consistent with this finding is that at present Hsa and SrpA are the only streptococcal SRR proteins that have been found to bind to GPIb $\alpha$ . Interestingly, a point mutation in the Siglec domain at R484E showed a marked reduction in binding to purified GPIb $\alpha$ , in addition use of this mutated streptococcus reduced vegetative growth in a rat model of

infective endocarditis [45]. *S. aureus* is also a common commensal of the human found predominately in the anterior nares of the nostrils and a opportunistic pathogen found in the blood stream. This bacteria also expresses a highly glycosylated SRR protein named SraP on its surface. A strain of *Staphylococcus aureus* deficient in expression of SraP led to the reduced virulence in a rabbit model of endocarditis [46]. Regardless of the fact that SraP shares significant similarities with a number of other SRR's found in the streptococci that have been found to bind to platelet GPIb $\alpha$ , it is currently unclear as to whether SraP binds to this platelet receptor.

### 3.1.2. Indirect interaction with GPIb $\alpha$

Additional studies identified that bacterial interaction with platelets was abolished when plasma was removed, suggestive of the need for a plasma protein in the interaction. Subsequent studies identified that a number of bacteria bind vWf and bridge the bacteria to platelet GPIb $\alpha$ . For example, *S. aureus* expresses protein A (SpA) on its surface which binds to immobilised vWf under both static and shear based conditions which in turn bridges to platelet GPIb $\alpha$ . Site directed mutagenesis demonstrated that all five domains of SpA (A-E) can bind to the A1 domain of vWf with high affinity (low nM range) [47]. It is well established that GPIb $\alpha$  binding vWf can cross link to another platelet via GPIb $\alpha$  which represents agglutination rather than true platelet aggregation involving GPIIb/IIIa and fibrinogen. Additional experiments are required to establish if the protein A-vWf interaction represents agglutination or true aggregation. *Helicobacter pylori* is the main causative organism of peptic ulcers and have been shown to induce platelet aggregation *in vitro* by binding to vWf which in turn bridges to platelet GPIb $\alpha$  triggering an activating response. Blocking vWf or GPIb $\alpha$  with inhibitory antibodies prevented the interaction. Patients lacking expression of GPIb $\alpha$  (Bernard Soulier Syndrome) fail to aggregate in response to *H. pylori* [34]. To date the *H. pylori* component that binds vWf has not yet been identified [48].

## 3.2. Glycoprotein IIb/IIIa

### 3.2.1. Direct interaction with GPIIb/IIIa

A number of different species of bacteria have been shown to bind directly to GPIIb/IIIa. Physiological ligands mediate attachment to GPIIb/IIIa via a short amino acid sequence, RGD. Consistent with this observation, several bacterial proteins have been identified to express an RGD-like sequence in their cell wall proteins. The serine/aspartate (SD) repeat family of proteins are among the most important cell wall components expressed on the surface of the skin commensal *Staphylococcus epidermidis*. SdrG, also referred to as Fbe, is expressed on up to 91% of clinical *S. epidermidis* strains [49]. Protein analysis identified a potential RGD-like sequence (<sup>643</sup>RTD) in the B-domain of the SdrG protein. Platelet adhesion to a purified recombinant B-domain of SdrG was inhibited by using either a short synthetic peptide containing the RGD sequence or the GPIIb/IIIa antagonist tirofiban (which spans the RGD site), suggesting that this sequence is responsible for directly interacting with GPIIb/IIIa [50].

Under iron limited conditions *S. aureus* expresses a surface determinant called IsdB which also contains an RGD-like sequence (<sup>248</sup>KYD) [51]. Surface plasmon resonance demonstrated that

recombinant IsdB interacts directly with purified GPIIb/IIIa with high affinity with a dissociation constant ( $K_D$ ) of  $405 \pm 73.7$  nM. Platelet adhesion and aggregation was significantly reduced following preincubation of platelets with the GPIIbIIIa antagonist tirofiban or an inhibitory RGDS peptide mimetic. Furthermore, a strain defective in expression of IsdB also failed to bind to GPIIbIIIa [51].

*Streptococcus gordonii* expresses a large molecular weight protein on its surface (397kDa) designated platelet adherence protein A (PadA). Platelet adhesion to *S. gordonii* was inhibited by the GPIIbIIIa antagonist abciximab or an inhibitory peptide mimetic, RGDS [52]. Platelets adhering to immobilised *S. gordonii* or specific fragments of PadA underwent dramatic changes in morphology as observed by fluorescent confocal microscopy. Rearrangement of the platelet actin cytoskeleton led to filopodia and lamellipodia formation resulting in full platelet spreading [53]. Proteomic analysis identified that PadA contains integrin recognition motifs ( $^{383}$ RGT and  $^{484}$ AGD) that may act as binding sites for GPIIbIIIa. Using site directed mutagenesis the AGD or RGT sequence was replaced with a AAA. Platelet interaction with these mutants demonstrated that platelet adhesion was unaffected however platelet spreading was significantly reduced. In addition, replacement of the RGT sequence to AAA (but not the AGD sequence) significantly reduced granule secretion [54]. These results suggesting that there are potentially multiple sequences on PadA responsible for specific platelet functions. The sequence that is directly responsible for supporting platelet adhesion has currently not yet been identified.

### 3.2.2. Indirect interaction with GPIIbIIIa

Both staphylococci and streptococci express a number of plasma protein binding proteins on their surface. Probably the most common are fibrinogen binding proteins, often expressed at different phase of bacterial growth. For example, *S. aureus* expresses clumping factor B (ClfB), fibronectin binding protein A (FnBPA), fibronectin binding protein B (FnBPB) in the early stage of growth (exponential phase) and clumping factor A (ClfA) in the late stage of growth (stationary phase). *S. epidermidis* expresses a fibrinogen binding protein, SdrG, at the exponential phase of growth. Group A (*Streptococcus pyogenes*) and Group B (*Streptococcus agalactiae*) streptococcus also express fibrinogen binding proteins called M protein and FbsA, respectively, in the exponential phase of growth.

The ligand binding sites of ClfA and ClfB have been mapped to residues 220 to 559 [55]. Interestingly the ligand binding sites of the two homologs are only 27% identical. In contrast to ClfA which recognises the extreme C-terminus of the  $\gamma$ -chain of fibrinogen, ClfB recognises the  $\alpha$ -chain of fibrinogen [56, 57]. The FnBPA or FnBPB can bind either fibronectin or fibrinogen. The N-terminal region of the fibronectin binding proteins (N1, N2 and N3) is structurally and functionally similar to the clumping factors, however in place of the serine-aspartate repeat region are tandemly repeated fibronectin-binding repeat domains. The FnBP's bind fibrinogen via the N1, N2 and N3 domains and fibronectin via the repeat domains (11 in FnBPA and 10 in FnBPB) [58]. SdrG (*S. epidermidis*) has the same structural organisation as the clumping factors and bind directly to the beta-chain of the fibrinogen molecule with a  $K_d$  in the range of 90-300 nM [59, 60].

Group A streptococci (*Streptococcus pyogenes*) express more than 80 types of the highly virulent factor, M-protein [61]. The fibrinogen binding motif differs between homologs of M-proteins, suggesting that this domain might have evolved independently in different M-protein lineages [62]. The exact binding site on fibrinogen that interacts with M-protein has not yet been defined, however in inhibitory peptide mimetic RGDS, abolishes platelet aggregation, suggesting the RGD site is involved [63]. Group B streptococcus (GBS) express three homologous proteins termed FbsA, FbsB and FbsC (Bsab). While all of these proteins have all been shown to bind fibrinogen to date only fsbA has been shown to be capable of binding fibrinogen and cross-linking to GPIIbIIIa [64]. Different GBS strains possess different numbers of repeat domains in the FbsA protein thus accounting for the size heterogeneity of the protein. Studies have demonstrated that a single repeat unit was capable of binding fibrinogen [65]. Although the site on the fibrinogen molecule that binds to FsbA was not identified, an RGDS peptide abolished the interaction suggesting that this motif on fibrinogen is critical [64].

A common observation is beginning to unfold in the light of all the fibrinogen binding proteins expressed on bacteria. Results demonstrate that where a bacterial protein binds fibrinogen and crosslinks to platelet GPIIbIIIa it is usually not enough to trigger an activating signal in the platelet and usually requires a co-stimulus. In all cases outlined above the key co-stimulus is provided by the bacteria binding IgG and cross linking to its reciprocal receptor on platelets, FcγRIIa.

### 3.3. FcγRIIA

#### 3.3.1. Direct interaction with FcγRIIA

Currently there are no reports of a bacterial protein binding directly to FcγRIIa on platelets, however there are a number of reports of an indirect interaction where bacterial proteins use IgG to cross link to platelet FcγRIIA.

#### 3.3.2. Indirect interaction with FcγRIIA

FcγRIIA is fast becoming the most important receptor in platelet bacterial interactions as it has been shown to inhibit all bacterial induced platelet activation including those triggered by *S. aureus*, *S. epidermidis*, *S. sanguinis*, *S. gordonii*, *Streptococcus pneumoniae*, *Streptococcus oralis*, *H. pylori* and *S. pyogenes* [30, 33, 34, 36, 50, 53, 66-70]. A key observation is that IgG is required for all of these bacteria to induce platelet aggregation, however antibody alone was not enough to trigger aggregation / activation of platelets. In all cases engagement of another platelet receptor was required such as GPIIbIIIa, GPIbα, or Toll Like Receptors (TLR's). These observations suggest that FcγRIIa requires receptor clustering in order to trigger platelet activation [69]. Indeed FcγRIIa has been shown to be physically associated with GPIIbIIIa and GPIbα and therefore plays an important role in their respective signalling in an IgG-independent manner [71].

Platelet receptor	Bacteria & protein	Bridging protein	Reference
GPIb $\alpha$	<i>S. gordonii</i> GspB/Hsa	Direct	42,43,44
	<i>S. sanguinis</i> SrpA	Direct	30,94
	<i>S. aureus</i> SraP	?	46
	<i>S. aureus</i> SpA	vWf	47
	<i>H. pylori</i> [unidentified]	vWf	48
GPIIb/IIIa	<i>S. epidermidis</i> SdrG	Direct	49,50
	<i>S. aureus</i> IsdB	Direct	51
	<i>S. gordonii</i> PadA	Direct	52,53,54
	<i>S. aureus</i> ClfA	Fibrinogen	55,56
	<i>S. aureus</i> ClfB	Fibrinogen	57
	<i>S. aureus</i> FnbpA/B	Fibronectin	58
	<i>S. epidermidis</i> SdrG	Fibrinogen	59,60
	<i>S. pyogenes</i> M1	Fibrinogen	62,63
	<i>S. agalactiae</i> Fbs	Fibrinogen	64,65
	<i>S. aureus</i> FnbpA/B	IgG	33
	<i>S. aureus</i> ClfA	IgG	36
Fc $\gamma$ RIIa	<i>S. aureus</i> ClfB	IgG	72
	<i>S. epidermidis</i> SdrG	IgG	50
	<i>H. pylori</i> [unidentified]	IgG	34
	<i>P. gingivalis</i> [unidentified]	IgG	73
	<i>S. aureus</i> SpA	direct	74,75
	<i>S. aureus</i> ClfA	?	32, 36
gC1q-R/P32	<i>S. aureus</i> ClfB	?	32,72
	<i>S. sanguinis</i> [unidentified]	?	38,77
	<i>S. pneumoniae</i> [unidentified]	direct	67
TLR2	<i>P. gingivalis</i> [unidentified]	direct	79

**Table 1.** Summary of interactions between bacteria and platelets. Bacteria can either interact with platelets directly or indirectly using a bridging protein, thus triggering activation. ClfA; clumping factor A, FnbpA; fibronectin binding protein A, SpA; protein A, PadA; platelet adhesion protein A, IsdB; iron-regulated surface determinant B, SdrG; Serine aspartate repeat G, Hsa; haemagglutinin salivary antigen, GspB; glycosylated streptococcal protein B, SrpA; serine rich protein A, IgG; immunoglobulin G, vWf; vonWillebrand Factor, C1q; complement 1q, GP; glycoprotein, TLR; Toll like receptor

Platelet receptor	Bacteria	Bacterial toxin	Reference
TLR4	<i>E. coli</i>	LPS	81,82,83,84
?	<i>S. epidermidis</i>	LTA	85,86,87,88
?	<i>P. gingivalis</i>	Gingipains	39,89
Glycospingolipids	<i>E.coli</i>	Verotoxin	41,90
?	<i>S. aureus</i>	$\alpha$ -toxin	91,92

**Table 2.** Summary of interactions between platelets and bacterial toxins. LPS; Lipopolysaccharide, LTA; Lipoteichoic acid

*S. aureus* and *S. epidermidis* induce platelet aggregation in an Fc $\gamma$ RIIa dependent manner. *S. aureus* fails to induce platelet aggregation in the absence of plasma proteins. Addition of IgG alone is not enough to trigger aggregation induced by *S. aureus*, however addition of fibrinogen/fibronectin and IgG combined induces full aggregation [33, 36, 72]. Similarly *S. epidermidis* SdrG also induces platelet aggregation however requires concomitant binding of fibrinogen and IgG to the A-domain of SdrG and an unidentified cell wall component of *H. pylori* requires concomitant binding of vWf and IgG which crosslinks to GPIb $\alpha$  and Fc $\gamma$ RIIa [34, 50].

All published reports of streptococcal induced platelet aggregation demonstrate their ability to induce platelet aggregation in an Fc $\gamma$ RIIa dependent manner. Early reports suggested that streptococci could induce platelet aggregation in the absence of plasma proteins. However regardless of this, blocking Fc $\gamma$ RIIa with a monoclonal antibody still abolished aggregation [30], suggesting that Fc $\gamma$ RIIa may be playing a role in signal amplification. This observation is analogous to the role of Fc $\gamma$ RIIa in promoting cell signalling/amplification through various platelets receptors such as GPIIbIIIa and GPIb $\alpha$ . Another oral bacteria, *S. oralis* on the other hand failed to induce platelet aggregation in gel filtered platelets [68]. The addition of plasma concentrations of fibrinogen to the gel filtered platelets failed to restore aggregation however addition of plasma concentrations of purified IgG fully restored platelet aggregation. More recently, Arman et al, demonstrated that *S. sanguinis*, *S. gordonii* but not *S. pneumoniae* require IgG's to induce platelet aggregation [69]. The site that IgG binds to was not identified in this study.

Oral bacteria, *Porphyromonas gingivalis* expresses a surface protein called hgp44 which induces platelet aggregation in an Fc $\gamma$ RIIa dependent manner [73]. The authors demonstrated that IgG was critical for the interaction. Preincubation of platelets with an anti-Fc $\gamma$ RIIa antibody abolished *P. gingivalis* induced platelet aggregation. Furthermore, depletion of IgG from plasma also completely prevented aggregation. Interestingly recombinant hgp44 failed to induce platelet aggregation suggesting that hgp44 may be dependent on another *P. gingivalis* protein binding to platelets in order to crosslink receptors to trigger a signal that results in platelet aggregation.

### 3.4. gC1q-R/P33

#### 3.4.1. Direct interaction with gC1q-R/P33

*S. aureus* SpA has been shown to be capable of binding directly to full length recombinant gC1qR (amino acids 1-282). Under resting conditions there is a low level of gC1qR expression on platelets, however upon activation expression levels significantly rises [74]. The latter suggests that another interaction between *S. aureus* and the platelet is required in order to express high levels of the receptor on the platelet surface. Binding was inhibited by both recombinant gC1qR and soluble purified protein A, however complement proteins did not appear to be required as the binding interaction occurs in the absence of complement proteins. Deletion of the C1q binding domain (amino acids 74-95) on the recombinant gC1qR resulted in loss of C1q binding but not *S. aureus* SpA suggesting that the SpA binding site is likely to reside outside of this domain [75].

#### 3.4.2. Indirect interaction

Complement is part of the immune system that augments the opsonisation of bacteria by antibodies which in turn facilitates phagocytosis. There are three main pathways that lead to complement activation; the classical pathway can be triggered by antigen-antibody complexes; the alternative pathway can be triggered by binding specific complement proteins binding to the bacterial surface and finally the lectin pathway can be triggered by mannose binding protein binding the bacterial surface [76]. The lag time to platelet aggregation in response to *S. aureus* (mediated by ClfA or ClfB) is 2-4 mins and required the binding of fibrinogen and IgG to their respective receptors on platelets in order to trigger full activation and aggregation [32]. Subsequent studies on these interactions discovered that by deleting the fibrinogen binding domain on *S. aureus* ClfA (ClfA-PY) or ClfB (Q235A) it significantly extended the lag time to aggregation (12-15 mins) suggestive of a second much slower interaction [36, 72]. Heating plasma to destroy proteins abolished platelet activation and aggregation by both ClfA-PY and ClfB-Q235A. Further studies using zymosan, which removes complement proteins from plasma, also inhibited platelet activation and aggregation by both ClfA-PY and ClfB-Q235A. These studies suggested that complement played a key role in the second much slower interaction that resulted in platelet activation/aggregation. The receptor on platelets that binds bacteria coated complement proteins has not been identified yet.

The lag time to platelet aggregation varies with different strains of *S. sanguinis* [77], for example *S. sanguinis* can induce platelet aggregation with a lag time of 2-4 minutes or 12-15 minutes. The long lag time is indicative of the time taken for complement assembly [38]. Inactivation of complement by cobra venom or heat treatment abolished aggregation [38]. It is not known precisely how complement activation triggers platelet activation but it is possible that there is a threshold of bacterial-platelet interactions (capable of inducing strong or weak signals) which must be surpassed before triggering platelet aggregation however this remains to be investigated.

### 3.5. Toll like receptor 2

#### 3.5.1. Direct interaction with TLR2

*Streptococcus pneumoniae* is a major pathogen usually found colonising the upper respiratory tract and nasopharynx. Following intense inflammatory response of the lungs the bacteria can gain entry to the bloodstream. *S. pneumoniae* has been shown to bind induce platelet aggregation and dense granule secretion [67]. Preincubation of platelets with an inhibitory TLR2 antibody abolished platelet aggregation and dense granule secretion induced by *S. pneumoniae*. Both aggregation and dense granule secretion was dependent on FcγRIIa however antibody was not required for either to take place, further suggesting that this receptor plays a role in signal amplification possibly through recruitment of adapter proteins to initiate signaling. Interestingly stimulation of TLR2 by *S. pneumoniae* resulted in the activation of the PI3 kinase pathway [67]. PI3-kinase is typically a negative regulator of TLR2-dependent responses in several immune cells. However, in platelets many studies demonstrate that PI3-kinase acts as a positive regulator in various platelet functions such as adhesion, aggregation and spreading [78]. Therefore it appears that PI3 kinase is a positive regulator of *S. pneumoniae*-induced platelet aggregation. Oral bacteria, *Porphyromonas gingivalis* induced platelet-neutrophil aggregates in a TLR2 dependent manner. This response was significantly reduced in TLR2 deficient mice, highlighting the importance of this receptor in the platelet recognition of *P. gingivalis* [79]. Consistent with previous observations the authors also demonstrated that PI3 kinase was critically important in the signalling response mediated downstream of TLR2.

## 4. Secreted products

Lipopolysaccharide (LPS) is shed from the cell wall of gram negative bacteria into the local milieu and interacts with Toll-like receptors (TLR) on immune cells [80]. *Escherichia coli* LPS was shown to bind to and mediate activation of platelets in a TLR4-dependent manner [81] and to enhance platelet secretion of cytokines [82]. Chicken thymocytes were also shown to express TLR4 receptor and to become activated by LPS [83]. There seems to be variation in the ability of different types of LPS to bind to platelets and LPS from *E. coli* O157 appears to be the most potent [81]. Although LPS was found to bind to TLR4 on platelets, there is little evidence to suggest that binding leads to platelet activation. LPS bound platelets had increased affinity for neutrophils and only LPS-treated platelets were capable of inducing neutrophil activation [84]. LPS injected into wildtype mice induced thrombocytopenia that was neutrophil-dependent [13]. Thus, current understanding is that LPS binds to platelet TLR4 but does not generate an activation signal, at least not one than leads to platelet aggregation. However, LPS appears to prime the platelets allowing them to bind to and activate neutrophils.

In contrast Lipoteichoic acid (LTA) is secreted by Gram-positive bacteria. LTA binds to platelets and inhibits platelet aggregation by collagen [85]. LTA also supports platelet adhesion to *S. epidermidis* [86]. Some studies suggested that the anti-platelet effect of LTA was due to conformational changes in the membrane [87] and an increase in cAMP levels [88].

In a manner similar to thrombin activation of the Protease Activated Receptors on the platelet surface, *Porphyromonas gingivalis* secretes gingipains which are proteases that can directly activate platelets [39, 89]. Shiga-like toxin (verotoxin) is secreted by *E. coli* and triggers platelet aggregation [90] by binding to glycosphingolipid receptors on the platelet surface [41].  $\alpha$ -toxin is a pore forming toxin produced by *S. aureus* which is responsible for haemolysis. It also leads to platelet activation [91] leading to the assembly of the pro-thrombinase complex on the platelet surface [92].

## 5. Animal studies versus clinically relevant models of infection

There are many reports in the literature investigating the interaction between bacteria and platelets *in vivo*. Translating animal studies to human disease is becoming increasingly difficult, especially with the identification that Fc $\gamma$ RIIA is a key receptor involved in signal amplification in human platelets [69]. Fc $\gamma$ RIIa is unique to higher primates and therefore calls into question the validity of using mouse, rat or rabbit animal models for studying platelet-bacterial interactions [93], as they do not express Fc $\gamma$ RIIa. Although mouse, rat and rabbit platelets do aggregate in response to bacteria *in vivo*, the mechanism through which they signal must be different. Transgenic mice expressing human Fc $\gamma$ RIIa are available commercially which can overcome this problem.

One potential possibility to overcome this problem is to develop a more clinically relevant model of infection using physiological conditions with human platelets. Using a parallel flow chamber with human platelets and shear conditions experienced under human physiological conditions a number of key interactions were observed. Under fluid shear conditions, human platelets rolled on immobilised *S. sanguinis* and *S. gordonii* at low shear rates (50s<sup>-1</sup>) followed by firm adhesion [42, 94]. This rolling behaviour was similar to the interaction between platelet GPIb $\alpha$  and vWf. Of key interest is that platelet rolling on vWf occurs under high shear conditions (>1000s<sup>-1</sup>) where the platelet rolling on bacteria occurs under low shear conditions (50s<sup>-1</sup>). Deletion of cell wall proteins *S. sanguinis* SrpA or *S. gordonii* Hsa abolished the rolling behaviour suggesting that these proteins must be in a conformation for direct interaction with platelet GPIb $\alpha$  under low shear conditions [42, 94]. Platelets rolled, adhered and formed micro thrombi on immobilised *S. oralis* at shear rates of 50s<sup>-1</sup> to 800s<sup>-1</sup>. This interaction was also dependent on GPIb $\alpha$  as determined by anti-GPIb $\alpha$  antibodies and blood from a Bernard Soulier patient. Aggregate formation was dependent on *S. oralis* binding IgG, which cross-links to platelet Fc $\gamma$ RIIa. This interaction also led to phosphorylation of the ITAM domain on Fc $\gamma$ RIIa, resulting in dense granule secretion and amplification through the ADP receptor [68].

*S. pyogenes* interacts with platelets under high shear conditions. The major cell wall component M-protein was found to bind IgG and fibrinogen which cross link to Fc $\gamma$ RIIa and GPIIb/IIIa respectively to trigger thrombus formation [95]. *S. aureus* also triggered thrombus formation in the presence of human whole blood under high shear conditions (800s<sup>-1</sup>). These studies identified that deletion of major cell wall protein ClfA but not other cell wall proteins (FnBP's, SpA or SdrC) completely abolished thrombus formation. Thrombus formation was dependent

on fibrinogen and IgG binding to platelets in order to trigger thrombus formation. Subsequent studies demonstrated that *S. aureus* ClfA bound IgG and fibrinogen crosslinked to Fc $\gamma$ RIIa and GPIIbIIIa respectively thus triggering thrombus formation. Of particular interest is that *S. aureus* failed to interact with platelets under low shear [66].

## 6. Conclusion

Although the field of platelet bacterial interactions is in its infancy, significant advances have been made in identifying some of the molecular mechanisms. Through learning about these interactions it has provided strong evidence that platelets may indeed be acting as primitive immune cells. However a lot more research is required to gain a better understanding of the exact role platelets play in in the process. For example, by adhering, aggregating, spreading or forming a thrombus on the bacteria are the platelets trying to restrict spread of infection and then by releasing their granular contents orchestrate or control the immune response to the infection by recruiting defined numbers of leukocytes. Alternatively is it a clever move by the bacteria who coat themselves in non-professional immune cells (platelets) and therefore rendering themselves safe from attack from professional immune cells (leukocytes) and antibiotics, which cannot penetrate the platelet encapsulation to kill the bacteria thus allowing them to grow and divide in a safe environment.

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