Chapter 2

Platelet-Bacterial Interactions in the Pathogenesis of Infective Endocarditis — Part I: The Streptococcus

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Additional information is available at the end of the chapter

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1. Introduction

Infective endocarditis (IE) is a life threatening disease caused by a bacterial infection of the endocardial surfaces of the heart. It is typified by the formation of septic thrombi or vegetative growth on the heart valve. Typically, both platelets and fibrin are deposited on exposed extracellular matrix proteins as part of the normal response to damage of the endocardium [1]. However, this sterile platelet-fibrin nidus facilitates colonisation of the endocardium by bacteria in the bloodstream [2]. Following attachment, bacteria can recruit platelets from the circulation inducing platelet activation and platelet aggregation. This results in the development of large macroscopic vegetations which resist infiltration by both immune cells and antibiotics making IE a difficult disease to treat. These vegetations commonly occur on the heart valves and can disrupt hemodynamic patterns within the heart. This puts undue force on often already compromised valves, leading to congestive heart failure [3]. IE is notoriously difficult to treat, requiring aggressive multi-antibiotic therapy often coupled with surgery to remove vegetations and/or replace the infected valve [4]. Therapy is successful when all traces of bacteria are absent from the blood stream. Multiple species of bacteria have been isolated from the infected vegetations of patients [5, 6] with IE but the streptococci are amongst the most common cause, second only to the staphylococci whose interactions with human platelets are discussed elsewhere in this book (Chapter X). Indeed, in a recent prospective study, the role of streptococci in IE is masked by the growing incidence of staphylococcal IE resulting from the increased use of medical procedures leaving streptococci as a main cause of IE in the normal population [7, 8].

2. The Streptococcus

The streptococci are a large family of gram positive coccus shaped bacteria that reside in the mouth, intestine, upper respiratory tract and the skin. Most have a commensal relationship

with their host. However, as opportunistic pathogens they can cause disease if they gain access to normally sterile sites of the body such as the bloodstream. Most streptococci isolated from patients with IE are of oral origin [9], normally found colonising the salivary tooth pellicle through the interactions of surface expressed virulence factors, called adhesins, with specific moieties or motifs on host proteins or cells. When these streptococci enter the bloodstream these bacterial components participate in additional interactions with platelets. Whether this is by design or simply as a consequence of conserved motifs within the human host is unknown but regardless their interaction with human platelets is a key step in the pathogenesis of IE.

3. Platelet biology I: Haemostatic function

In the absence of infection, platelets act as sentinels of vascular integrity, patrolling the endothelium for sites of damage. Upon vascular damage these small anucleate cells interact with exposed extracellular matrix proteins via specific receptors expressed on their surface and a complex yet coordinated series of interactions and signalling events proceed, culminating in the formation of a haemostatic plug. Platelet receptor complex GPIb-IX-V recognises von Willebrand Factor (vWF) bound to exposed collagen fibrils in the subendothelial matrix, tethering the platelet to the site of damage [10, 11]. This initial interaction is relatively weak and has a fast on-off rate [12] so the platelet characteristically rolls along the endothelium breaking and remaking the vWF-GPIb-IV-X interaction [1]. This 'rolling' mechanism slows the platelet sufficiently for additional receptor-ligand contacts and triggers an intracellular signal resulting in integrin activation and firm adhesion. Firm adhesion is mediated by a combination of ligand receptor engagements: integrin $\alpha 2\beta 1$ - collagen [13-15]; glycoprotein GPVI - collagen [16]; $\alpha 5\beta 1$-fibronectin [17]; and $\alpha IIb\beta 3$ with fibrinogen and vWF [12, 18]. Once firmly adhered, the platelet undergoes dramatic rearrangement of its cytoskeleton causing platelet shape change from its resting discoid form to a dendritic form and finally, to a fully spread platelet with characteristic filipodia and llamelipodia [19]. During this process, the platelet secretes signalling molecules, proteins and platelet agonists (ADP, ATP and serotonin) from its cytoplasmic granules (α- and dense- granules) and synthesizes and secretes thromboxane, amplifying the platelet response, recruiting and activating nearby platelets. Activated platelets can undergo platelet aggregation, cross linking with one another via their $\alpha IIb\beta 3$ receptors and the divalent plasma protein fibrinogen. This activation and recruitment of platelets to the site of injury, in addition to stimulation of the coagulation system and formation of a fibrin network, forms the haemostatic plug.

4. Platelet biology II: Immune function

The platelet role in haemostasis and thrombosis is well characterised but, at the same time, their activities can be viewed from an immunological perspective [20]. Their primary role of maintaining vascular integrity is an essential process in preventing entry of foreign particles into the blood stream. The epithelial barrier performs a similar function. More specifically,

however, they produce antimicrobial peptides [21]; possess pathogen recognition receptors (TLRs)[22, 23]; secrete immunomodulatory molecules [24]; and have specific receptors for chemokines [25], antibody complexes [26] and complement factors [27]. Critically, as outlined in this chapter, they interact with and respond to bacteria, all hallmarks of true immune cells. It is this response, or activation, that is important in the context of IE and provides the basis for the formation of platelet-bacterial vegetations which characterise this disease.

5. Platelet-bacterial interactions: General observations

Platelet interactions are examined under the broad headings of platelet adhesion, activation and aggregation. Platelet aggregometry is a useful tool in assessing platelet activation. In contrast to conventional stimuli such as ADP and thrombin, there is a significant lag time to the onset of platelet aggregation in response to bacteria [28].The length of this lag is defined by the sum of platelet-bacterial interactions occurring and can vary between donors, most likely due to variation in the levels of platelet receptors expressed on the surface and the concentration of plasma proteins. Platelet-bacterial interactions can be categorized into direct, indirect or mediated by a secreted bacterial product [29]. A direct interaction occurs when a bacterial adhesin binds directly to a platelet receptor or other surface expressed component [30]. Bacteria can participate in indirect interactions with platelets through a bridging protein which binds to the bacterium and then to its cognate platelet receptor [31]. When bacteria enter the bloodstream they can bind plasma proteins through specific plasma binding proteins or they can simply be recognised by soluble elements of the immune system such as immunoglobulins and complement proteins. Finally, and less common for the streptococcal bacteria, a secreted bacterial product may bind to the platelet causing activation independently of bacterial attachment. The ability of bacteria to propagate platelet activation and aggregation facilitates growth of the vegetation and effectively encapsulates the bacteria, hiding them from conventional immune cells and bacterial killing mechanisms. This chapter will focus on the specific molecular events that lead to initiation of IE, namely recognition of the platelet by the bacteria (and vice versa) and the ensuing intracellular signalling events that lead to platelet activation and amplification of the platelet response. As will be evident from the discussion to follow, platelet bacterial interactions are heterogeneous in nature and additionally they are multifactorial, with most bacteria interacting with platelets through more than one mechanism.

6. Streptococcus — Platelet interactions

6.1. Early findings

Streptococcus sanguinis (formally *Streptococcus sanguis*) is the most common *Streptococcus* isolated from the valves of patients with IE [9]. Early studies identified a 115 kDa cell membrane protein that induced platelet aggregation in platelet rich plasma [32]. This rhamnose rich,

platelet-aggregation associated protein (PAAP) is glycosylated and was amongst the first identified bacterial glycoproteins [33]. Bacterial glycoproteins are now thought to be almost ubiquitous, performing critical roles in host adhesion, resistance to complement killing, maintenance of cell shape and enzymatic activities that release nutrients from complex carbohydrates [34]. The platelet receptor for this protein has not been confirmed, however, an early study showed that *S. sanguinis* did not induce platelet aggregation in a patient who failed to respond to collagen suggesting the role of a collagen receptor, possibly $\alpha_2\beta_1$ [35]. Additionally, Gong et al. isolated platelet membrane proteins of molecular weights 175 kDa, 150 kDa and 230 kDa that interacted with *S. sanguinis* 133-79 [36]. The platelet binding domain of PAAP has been isolated to a 23 kDa fragment [37]. Furthermore, the peptide sequence PGEQGPK within this fragment conforms to the platelet interactive domain of collagen types I and III, KPGEPGPK, and antibodies directed against this peptide delayed the onset of aggregation induced by *S. sanguinis* [38, 39]. More recently, in an effort to identify the PAAP gene, a putative collagen binding protein was identified containing two PAAP-like sequences and platelet aggregation in platelet rich plasma was significantly reduced in response to a mutant lacking this protein while no changes in platelet adhesion were observed [40]. In conjunction with the study of Gong et al., this confirmed that *S. sanguinis* had at least one other adhesin for human platelets.

6.2. Serine rich repeat glycoproteins

The serine rich repeat (SRR) proteins are a large family of glycosylated bacterial adhesins. SRR proteins of *S. sanguinis* and its close relative *S. gordonii* mediate direct binding of these bacteria to platelets through sialic acid residues on the GPIbα subunit of the GPIb-V-IX complex [41-43].

Fimbriae-associated protein 1 (Fap1) of *S. parasanguinis* FW213 was the first SRR protein to be identified and, while it is reported not to interact with human platelets, studies of Fap1 have provided important information on the structure of SRR proteins and the nature of their ligand interactions. SRR proteins share a common domain structure: an N-terminal signal sequence; a short serine rich repeat region (SR1); a non-repetitive ligand binding region (BR); a larger serine rich repeat domain (SR2); and a cell wall anchor domain (CW) [41-47]. Like PAAP, SRR proteins are highly glycosylated. The serine rich repeat domains are decorated with O-linked carbohydrate residues [48] and the larger SR2 domain is thought to form a stalk like structure, extending the adhesive N-terminal region from the cell surface. Fap1 is critical for *S. parasanguinis* adhesion to saliva coated hydroxyapatite (sHA) and biofilm formation [49, 50]. However, Fap1 mediated adhesion to sHA is independent of these glycosylations as shown by mutation of upstream glycosyltransferases critical for glycosylation of the native protein and subsequent biofilm formation [51].

In contrast to the conserved structural organisation of SRR proteins, the peptide sequence of the non repetitive region varies significantly and is suggested to explain the differing affinity of SRR proteins to platelets and other cell types. To date only the SRR proteins of *S. sanguinis* (SrpA) and *S. gordonii* (GspB and Hsa) have been demonstrated to interact directly with human platelets while others, PsrP of *S. pneumoniae* and Srr-1 of *S. agalactiae* bind to keratin 10 and 4 in lung epithelial and endothelial cells respectively [52, 53]. The platelet interactive

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domains and specifically the sialic acid binding domain of GspB and Hsa are isolated to the non repetitive region [43]. Most recently, x-ray crystallography studies of the non-repetitive ligand binding region of GspB have revealed a modular organization: helical domain; a domain similar to the binding domain of *Staphylococcus aureus* collagen binding protein CnaA; a Siglec domain; and a Unique domain [54]. Interestingly, the Siglec domain, a mammalian carbohydrate binding domain, was found in Hsa and SrpA but not in the protein sequences of five other characterised SRR proteins suggesting that this domain is critical for interactions with GPIbα [54]. Indeed, a point mutation (R484E) in the Siglec domain showed a marked reduction in binding to glycocalicin, the ectodomain of GPIbα and reduced bacterial load in the vegetations of a catheterized rat model of infective endocarditis [54].

GPIbα is a glycosylated, type one transmembrane receptor. A long highly glycosylated region called the macroglobulin region, or mucin-like core, extends from the cell surface presenting ligand binding domains to the extracellular milieu [55]. The macroglobulin region is decorated in predominantly O-linked but some N-linked carbohydrates terminating in sialic acid[55]. This highly glycosylated protein backbone is followed by a sulphated tyrosine region, a leucine rich repeat domain and an N-terminal domain decorated with N-linked sialic acid oligosaccharides [55]. Hsa is proposed to bind to both the N-terminal domain and the macroglobulin region while GspB interactions are isolated to the macroglobulin stalk [43]. Further complexity is added as GspB and Hsa display distinct preferences for O-linked and N-linked glycosylations respectively [43]. The subtle differences in binding affinity of SrpA to sialic acid moieties remain to be elucidated but studies using anti-GPIbα site specific antibodies isolated the *S. sanguinis* interactive region of GPIbα to the N-terminal region and the sulphated tyrosine region [30]. This suggests that SrpA interacts with human platelets in a distinct mechanism to GspB and Hsa. However, the ability to bind sialic acid residues is critical as sialidase treated platelets and glycocalicin support significantly less bacterial binding than the untreated samples [41-43]. In platelet function studies, deletion of either Hsa or SrpA failed to prevent platelet aggregation suggesting other platelet-bacterial interactions are needed to induce platelet activation [41, 56]. In contrast, using an *in vitro* model of blood flow, platelets were observed rolling before stably adhering to *S. sanguinis* 133-79 [41]. This is characteristic of GPIbα interactions with vWF under shear conditions. When platelets were perfused over an immobilised strain of *S. sanguinis* defective in expression of SrpA or an *S. gordonii* strain defective in Hsa expression, no rolling or attachment was observed suggesting that these SRR are essential for initial platelet attachment in the blood stream [41, 56].

7. Antigen I/II family of bacterial adhesins

The antigen I/II family of proteins are ubiquitous to streptococci being found in most published genomes to date with roles in the development of microbial communities and adhesion to host cells and proteins [57]. Like the SRR proteins they share a common domain organisation: a signal sequence; an N-terminal region; an alanine rich repeat domain; a variable domain; a proline rich repeat region; a C-terminal region and a cell wall anchor domain [58].

Investigation of the role of the antigen I/II family of adhesins in *S. gordonii*-platelet interactions was prompted by the observation that, while adhesion to *S. gordonii* DL1 was reduced by mutation of Hsa, the platelet aggregation response remained, suggesting a second interaction [56]. Indeed, a proteomic approach using cell wall extracts from an aggregating *S. gordonii* strain (DL1) and a non aggregating strain (Blackburn) revealed two antigen I/II proteins of molecular weights 172 kDa and 164 kDa [56]. These were designated SspA and SspB [56]. Mutation of these proteins did not affect platelet adhesion to wildtype *S. gordonii* or, indeed, a Hsa mutant. However, platelet aggregation was completely abolished when Hsa, SspA and SspB were mutated simultaneously [56, 59]. SspA and SspB participate in fluid phase interactions with salivary glycoprotein gp340, facilitating bacterial clumping which most likely aids in the development of biofilms [59, 60]. Additionally, they mediate adherence and internalisation into epithelial cells via β1 intregins [57], can bind to collagen type 1 [61] and interact with other oral microorganisms: *Candida albicans* [62]; *Porphyromonas gingivalis* [63]; and *Actinomyces naeslundii* [60]. Given their critical role in induction of platelet aggregation it is tempting to speculate that *S. gordonii* strains lacking antigen I/II proteins may have reduced virulence in IE due to failure to propagate platelet activation. However, this remains to be confirmed in animal models of IE.

The cariogenic and IE causing bacterium *S. mutans* also produces an antigen I/II adhesin called PAc, P1 or SpaA that has been shown to be involved in platelet aggregation [64]. PAc is a LPXTG cell wall associated protein with significant sequence identity to *S. gordonii* SspA [65]. PAc, like SspA and SspB, has roles in adherence to the salivary pellicle, biofilm formation [66], collagen dependent invasion of dentinal tubules binding [67]. Clinical strains lacking expression of PAc failed to induce aggregation in PRP [64]. Additionally, increasing amounts of anti-PAc serum dose dependently decreased the rate of platelet aggregation but did not abolish it [66]. A recent crystallography study examined the detailed structure of the C-terminal in the context of adherence to the salivary pellicle, specifically the binding of carbohydrate moieties [68], however, little is known about the putative platelet interactive domain of PAc. Notably, while antibody titres against PAc are increased in patients with *S. mutans* IE, PAc did not play a role in IE in a rat model of infection [69]. In contrast, a study examining the role of *S. mutans* exopolysaccharides revealed a substantial decrease in the incidence of IE in rats infected with wildtype *S. mutans* and a mutant lacking production of glucan and fructan polysaccharides [70]. Chia et al. later identified a direct interaction of *S. mutans* Xc rhamnose-glucose polymers (RGPs) with both human and rabbit platelets and showed the resulting platelet aggregation response to be mediated in part by anti-RGP IgGs [71]. Such rhamnose rich polymers are common amongst streptococcci and may represent a conserved mechanism of platelet activation by the *Streptococcus* genus [71].

8. High molecular weight repeat protein

Previous studies revealed that *S. gordonii* DL1 could bind to not only to platelet GPIbα but also GPIIb, the α chain of the fibrinogen binding integrin αIIbβ3 [72]. Further studies identified a large 397 kDa cell wall associated protein designated platelet adherence protein A (PadA) on

the surface of *S. gordonii* which interacts with GPIIb [73]. The N-terminal fragment of PadA contains a domain with homology to the A1 domain, the platelet interactive domain, of vWF. This, however, showed no particular affinity for the vWF receptor, GPIbα. An isogenic PadA mutant displayed the same affinity for glycocalicin as wildtype DL1 while binding was significantly reduced in a Hsa mutant [73]. In contrast, platelets adhered to immobilised fragments of the N-terminal region (amino acids 34-690) but not to a smaller fragment (amino acids 34-359) also containing the vWF domain suggesting other sites within the protein contribute to platelet adhesion to PadA [73]. Mutants lacking Hsa bound at wildtype levels to Chinese Hamster Ovary (CHO) cells expressing αIIbβ3 while a PadA mutant displayed significantly reduced adhesion. Additionally, CHO cell adhesion to wildtype bacteria was inhibited by a monoclonal antibody to αIIbβ3 (abciximab) and a fibrinopeptide mimetic, RGDS [73]. Interestingly, platelets adhering to immobilised *S. gordonii* DL1 or specific fragments of PadA underwent dramatic changes in morphology as observed by fluorescent confocal microscopy [74]. Rearrangement of the platelet actin cytoskeleton led filopodia and llamelipodia formation, known as platelet spreading [74]. Platelet spreading is critical for the platelet to withstand shear forces experienced in the vasculature. Similar observations were made for platelet adhesion to fibrinogen, suggesting that PadA mimics the prothrombotic surface of immobilised extracellular matrix proteins. Indeed, protein analysis revealed PadA contains RGD-like regions (RGG, RGT and AGD) that may act as binding sites for αIIbβ3 [74]. These observations have led to the model of *S. gordonii* platelet interactions: Hsa and GspB mediate initial attachment of *S. gordonii* to platelets via GPIbα; PadA provides stabilising interactions via αIIbβ3, causing platelet spreading, so that the growing septic thrombus can resist the turbulent forces within the bloodstream; and SspA and SspB are needed to produce the final aggregation phase that propagates vegetation growth [59].

9. Phage encoded proteins

Human and bacterial evolution is peppered with incidences of viral integration or endogenisation into host genomes. Indeed, when the human genome was sequenced it was found that only 1.5% was composed of defined genes [75, 76]. The remainder, formally referred to as "junk DNA", is now known to contain critical regulatory sequences. Many of these regulatory sequences and indeed genes have been linked to viral origins [77-79]. Bacterial history is also littered with incidences of viral DNA integration. Bacterial viruses are called bacteriophages and in the cases below they confer an advantage to *S. mitis* in the pathogenesis of IE.

Using a transposon generated mutant library of *S. mitis* SF100, two genetic loci were identified as having a role in *S. mitis*-platelet interactions [80]. The first, PblT, is predicted to encode a transmembrane transporter with 12 membrane spanning segments [80]. Its role in *S. mitis*-platelet interactions remains to be to be confirmed. Interestingly, the second locus was demonstrated to be a bacteriophage, SM1, of the *Siphoviridae* family of bacteriophages [81] and is widespread in the microbial population of the oropharynx and saliva as shown in a recent metagenomic study of oral viral communities [82]. Two proteins, PblA and PblB, encoded in the polycistronic operon of this phage were shown to mediate *S. mitis* binding to platelets [80,

83]. PblA and PblB are expressed on the bacterial surface through a novel mechanism whereby the proteins are exported and become associated with bacterial cells via choline residues in their cell wall [84]. While the ability to bind choline residues is found in other streptococcal expressed proteins (*S. pneumoniae* LytA), PblA and PblB bear little homology to previously identified bacterial adhesins [80]. Instead, they are similar to the tail fibre proteins of phage particles [80]. Recently a comprehensive study by Mitchell et al., utilizing linkage specific sialidases, concluded that PblA and PblB bind sialic acid residues on α2-8 linked gangliosides [85]. Consistent with this, platelets express only one such ganglioside, GD3, and specific antibodies to this receptor significantly reduced binding of wild type *S. mitis* SF100 to platelets while a mutant, with already significantly reduced in binding to platelets, remained unaffected [85]. The precise role of this receptor in conventional platelet activation remains to be determined but it has been shown to be upregulated on activated platelets, later becoming internalised and associating with the Src tyrosine kinase, Lyn, and then with FcRγ and leading to increased FcγRIIA expression [86]. How *S. mitis* would propagate platelet activation through this receptor remains to be elucidated.

Interestingly, during the investigation of PblA and PblB, a study revealed that mutation of the bacteriophage lysin gene, *lys*, needed to permeablise cells and release lytic phage particles, caused a reduction in platelet binding greater than that of the PblA$^-$/PblB$^-$ mutant [84]. When investigated further, the phage lysin was found to bind fibrinogen via the D fragment of the Aα and Bβ chains, and in doing so can mediate an indirect interaction with human platelets through αIIbβ3 [87]. Like PblA and PblB, it is also a choline binding protein but with homology to the choline binding domain of pneumococcal LytA [87]. The fibrinogen interactive domain was localized to amino acids 102-198 [88] and when this polypeptide was preincubated with platelets and *S. mitis* SF100, it significantly extended the lag time to aggregation. Furthermore, in a rat model of endocarditis, co-infection with PblA$^-$/PblB$^-$ and lys$^-$ mutants led to substantially less inclusion of lys$^-$ *S. mitis* in the vegetations as compared to the tail protein mutant, PblA$^-$/PblB$^-$. Lysin$_{SM1}$ is considered a multifunctional phage protein, mediating lysis of *S. mitis* in the bacteriophage lytic life cycle, binding to choline residues in the cell wall and binding to fibrinogen, bridging an interaction with human platelets via αIIbβ3 [87], an interaction that is repeated in multiple streptococcal species and considered an important interaction in the pathogenesis of IE (see figure).

10. Secreted products

Bacteria can secrete bioactive molecules that participate in platelet interactions *in trans*, independently of bacterial cell binding. Early studies examining the role of aetiological agents in Kawasaki disease (an inflammatory disease characterised by systemic vasculitis) isolated a strain of *S mitis* (Nm65) whose culture supernatant appeared to induce platelet aggregation in platelet rich plasma [89]. This activity was isolated to a heat labile, 66kDa protein antigen called sm-hPAF (*S. mitis* derived human platelet aggregation factor) [90]. Notably, 'aggregation' was not inhibited by treatment of platelets with prostaglandin E_1 (PGE_1), which increases intracellular cyclic-AMP preventing platelet signalling, or the αIIbβ3 inhibitory peptide (RGDS) and

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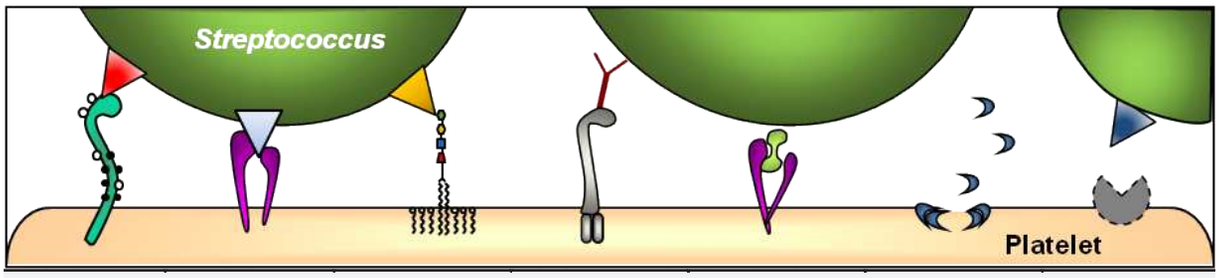

GPIbα	αIIbβ3 (direct)	Ganglioside GD3	IgG - FcγRIIA	Fibrinogen - αIIbβ3 (indirect)	?	Unknown receptors
S. gordonii (Hsa/GspB) *S. sanguinis* (SrpA)	*S. gordonii* (PadA)	*S. mitis* (PblA, PblB)	*S. sanguinis* *S. mitis* *S. mutans* *S. pyogenes* *S. agalactiae* *S. oralis*	*S. agalactiae* (FbsA) *S. mitis* ($Lysin_{SM1}$) *S. pyogenes* (M protein)	*S. mitis* (sm-hPAF/ lectinolysin)	*S. sanguinis* (PAAP) *S. gordonii* (SspA/SspB) *S. mutans* (PAc,RGPs)

Figure 1. Schematic of streptococcal platelet interactions in infective endocarditis.

was considered to induce platelet aggregation via novel mechanism [90]. With developments in our understanding of platelet aggregation, inhibition of platelet signalling and activation by PGE_1 and αIIbβ3 dependency are hallmarks of true platelet aggregation. Sm-hPAF (Nm-65 derived) and lectinolysin (SK597derived) were later purified independently and identified as members of the cholesterol dependent cytolysin (CDC) family of bacterial toxins which form oligomeric lytic pores in erythrocyte membranes [90, 91]. Both lectinolysin and Sm-hPAF possess an additional N-terminal fucose binding domain homologous to an agglutinin from the eel species *Anguilla Anguilla*. Interstingly, lectinolysin was demonstrated to induce pore formation via a mechanism modulated by fucosylated glycan binding to the N-terminal domain [91]. However, this domain did not participate in initial receptor recognition as lysis was detected in the absence of a functional glycan binding domain [91] and additional members of the CDC family lacking this domain, suislysin and pneumolysin, also induced platelet lysis [92]. The role of platelet lysis in IE remains to be established.

11. Streptococcal-platelet interactions — Signalling response

Following platelet activation platelets secrete signalling molecules and platelet agonists from their cytoplasmic granules to recruit and activate nearby platelets. *S. sanguinis* can modulate the platelet response through an unusual interaction whereby a surface associated enzyme can modify secreted platelet agonists. Early studies by Herzberg et al. demonstrated that *S. sanguinis* could hydrolyse exogenous ATP to ADP and this was postulated as a mechanism causing platelet aggregation as a cell free supernatant of *S. sanguinis* preincubated with ATP could induce platelet aggregation almost immediately [93]. Later MacFarlane et al. demonstrated that this ecto-ATPase activity localized to a cell wall fraction of *S. sanguinis* [94]. Fan et al. have recently identified a cell wall anchored ecto-5′ nucleotidase (Nt5e) from *S. sanguinis*

which can hydrolyse ATP, ADP and AMP producing adenosine [95]. A mutant lacking Nt5e expression had a shortened lag time to platelet aggregation with no effect on platelet adhesion but, interestingly, had decreased virulence in a rabbit model of IE as compared to the wildtype. This was suggested to be due to the inhibition of professional phagocytes, monocytes and macrophages by adenosine, an anti-inflammatory molecule [95]. In addition, the delay in platelet aggregation may delay the release of platelet microbicidal proteins from their granules thus inhibiting the platelet immune response [95].

As mentioned, platelets spread on *S. gordonii* DL1. While platelet spreading is critical for thrombus stability, dense granule secretion is important for amplification of the platelet response, facilitating activation of nearby platelets, recruiting them to the growing thrombus. Both result from initiation of an intracellular signalling cascade caused by PadA engagement of αIIbβ3 [74]. Interestingly, inhibition of platelet FcγRIIA by a monoclonal antibody (Clone IV.3) prevented both platelet spreading and dense granule secretion [74]. FcγRIIA is an ITAM containing receptor [96]. It has an extracellular domain that interacts with immune complexes and an intracellular domain which is proposed to act as a signalling scaffold allowing recruitment protein kinases and phosphatases. Following platelet adhesion and spreading on *S. gordonii* DL1, immunopercipitation of FcγRIIA and its downstream effectors revealed tyrosine phosphorylation of FcγRIIA, Syk and phospholipase Cγ (PLCγ – an effector of dense granule secretion) [74]. Blockade of FcγRIIA by the antibody IV.3 prevented phosphorylation of FcγRIIA and its downstream effectors while it had no effect on platelet adhesion to *S. gordonii* DL1 demonstrating an essential role in platelet activation and signalling but not in initial attachment to the bacterium.

Similarly, Pampolina et al. examined the phosphorylation state of the FcγRIIA and its downstream effectors during the platelet aggregation response to *S. sanguinis* 2017-78. *S. sanguinis* 2017-78 induced phosphorylation of FcγRIIA, Syk, Linker for activation of T-cells (LAT) and PLCγ 30 seconds after the addition of bacteria to the platelet suspension [97]. This was followed by dephosphorylation during the lag phase and αIIbβ3 and thromboxane dependent rephosphorylation as aggregation proceeded [97]. The MAP kinase Erk was observed to follow the same triphasic phosphorylation profile in response to *S. sanguinis* 2017-78 [98]. The dephosphorylation phase is proposed to be due to the activity of platelet endothelial cell adhesion molecule-1 (PECAM-1), an ITIM containing receptor which recruits the tyrosine phosphatase SHP-1 during the lag phase [97]. Further studies by McNicol et al. highlighted a role for PI3 kinase mediated phosphorylation of Erk in response to *S. sanguinis* 2017-78 [99]. PI3 kinase is found downstream of FcγRIIA and GPIb and upstream of the GTPase Rap1b, critical for αIIbβ3 activation [100, 101].

12. Streptococcal-platelet interactions — Immunological response

When bacteria enter the bloodstream they are recognised by soluble elements of the immune system. These soluble elements, specifically immunoglobulins and complement proteins can bind to their respective receptors on professional immune cells and platelets forming indirect

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bridging interactions. As most bacteria causing infective endocarditis are in fact lifelong or transient residents of the host, most of the population inherently produce a humoral immune response to these bacteria.

The role of IgG in platelet bacterial interactions has been extensively studied and reaffirmed repeatedly in the literature [28, 102-105]. Its role was first confirmed by Sullam et al. who demonstrated that plasma components other than fibrinogen (a cofactor for ADP induced activation) was required for *S. sanguinis* M99 and *S. salivarius* D1 induced platelet aggregation [104]. Additionally, blockade of the platelet low affinity IgG receptor, FcγRIIA, with a monoclonal antibody IV.3 completely inhibited platelet aggregation in response to these bacteria [104]. In fact, inhibition of FcγRIIA has inhibited all bacterial induced aggregation when examined, even in the absence of IgG interactions consolidating its role in platelet activation by bacteria [30, 74, 84, 97, 102, 106-108]. The nature of these antibodies, however, remains more complex as some bacteria require strain specific [107], species specific or, minimally, group specific antibodies [105]. Many conserved structural entities of bacteria elicit antibody responses e.g., peptidoglycan and lipoteichoic acids. Therefore, it is plausible that specific subclasses of antibody cross react between streptococcal species while others recognise species and strain specific antigens. Indeed, while IgG1 and IgG3 largely bind protein antigens, IgG2 binds to carbohydrate antigens [109]. Antibody levels in the host were first thought to determine the variable lag times observed between donors however this could not be established and led to investigations of other plasma proteins mediating platelet-bacterial interactions. Additionally, in a study of aggregating and non aggregating strains of *S. sanguinis* and *S. gordonii*, significant correlation between the levels of specific antibody and the propensity to induce aggregation was observed, but this was not true for all donors [105]. Notably, non aggregating strains and their non aggregating donor pairs were shown to have similar levels of strain specific antibody to donors who did support platelet aggregation [105]. This was not explained by polymorphisms in FcγRIIA and thus is most likely a result of secondary interactions with other receptors, differences in receptor number or polymorphisms therein but this remains to be investigated.

The complement system is a series of proteins that bind to bacteria in a step wise fashion and culminate in the formation of an oligomeric pore, the membrane attack complex, which lyses the targeted bacterium. Roles for complement in bacterial-platelet interactions have been demonstrated in *Staphylococcal aureus* and *S. sanguinis* [102, 107, 110]. The lag time to aggregation in platelet rich plasma (PRP) in response to *S. sanguinis* 7863 is 7-19 minutes [111] and this variation was correlated to the rate of assembly of the C5b-9 complex on the surface of the bacteria as detected by flow cytometry [110]. Accordingly, the lag time to aggregation using bacterial cells preincubated with plasma before addition to PRP could be progressively shortened with extension of the incubation time [110]. Complement activation can be triggered by antigen-antibody complexes (classical pathway) or by binding of specific complement proteins (alternative pathway) or mannose binding protein (lectin pathway) to the microbial surface. *S. sanguinis* 7863

induced the alternative pathway, as shown by direct and Mg2+ dependent binding of complement protein C3 to the surface of the bacterium [110]. Inactivation of complement by cobra venom or heat treatment abolished aggregation [110] suggesting that other interactions, namely IgG with FcγRIIA, were not sufficient to produce aggregation alone. 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 much be surpassed before triggering platelet aggregation however this remains to be investigated.

While previous reports examined platelet secretion in terms of end stage platelet activation, namely aggregation and spreading, a number of studies exist investigating the role of platelet secretion in the context of platelet immunology. In addition to platelet agonists, platelet granules contain bacteriocidal proteins and cytokines. A recent study by McNicol et al. demonstrated platelet activity in the form of signalling and secretion in the absence of platelet aggregation. They examined platelet secretion of soluble inflammatory mediators (Platelet factor 4, RANTES, sCD40L, platelet derived growth factor) in response to a number of *S. sanguinis* and *S. gordonii* strains and paired these with their platelet aggregation responses. All strains triggered secretion of cytokines irrespective of the platelet aggregation response but only 1 strain (*S. sanguinis* 2017-78) triggered release of sCD62p [99]. For *S. sanguinis* 2017-78 cytokine secretion was independent of thromboxane production and aggregation. Interestingly this secretion response was inhibited by low doses of epinephrine while aggregation and protein phosphorylation cascades mentioned previously were enhanced [99]. The inhibition of platelet activation by epinephrine has not been noted in response to any other platelet agonists and adds another layer of complexity to bacterial induced platelet activation. It will be interesting to examine the contribution of individual platelet-bacterial interactions this novel platelet activation in the future.

13. Conclusion

The overall role of platelet activation in response to circulating bacteria and IE is controversial but recent studies have linked platelet activation to the ability of bacteria to resist antibiotics [112]. This is consistent with the concept that, in activating platelets, bacteria prevent infiltration by antibiotics (or recognition by the immune system). It is likely that the initial adhesion events occur independently of platelet activation and thus make suitable targets in the prevention of IE. In contrast, as the ability to induce platelet aggregation (activation) *in vitro* contributes to the virulence and persistence of the organism in infective endocarditis animal models [87, 95], the pathways of platelet aggregation are targets of future IE therapies. Consistent with this, a recent study has examined the effect of antiplatelet drug Reopro (abciximab) in the treatment of sepsis in mice [113] and the use of cyclooxygenase inhibitors, e.g. aspirin and inbuprofen continue to be investigated [114-117]. Critically, future therapies must balance immune function and haemostatic function of platelets making thorough understanding of platelet-bacterial interactions and bacterial induced platelet activation essential for future drug development.

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