Bruton's Tyrosine Kinase Inhibitors Impair FcγRIIa-Mediated Platelet Responses to Bacteria in Chronic Lymphocytic Leukaemia

Leigh Naylor-Adamson

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University of Hull and University of York

Hull York Medical School

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Abstract:

Ibrutinib is highly effective in the treatment of chronic lymphocytic leukaemia (CLL), disrupting B cell receptor signalling through the inhibition of Bruton's tyrosine kinase (Btk). However, ibrutinib has off-target effects, inhibiting platelet aggregation with associated haemorrhage. More recently, second generation Btk inhibitors have been developed to be more specific for Btk, including acalabrutinib. Still, acalabrutinib is similarly associated with a bleeding risk. Treatment of CLL with ibrutinib is also connected to an increased infection risk.

Though platelets are known for their role in haemostasis, they additionally play a key role in innate immunity. Platelets can interact with bacteria through a variety of mechanisms, with one such mechanism being the FcγRIIa receptor, a low affinity IgG immune receptor that can mediate platelet aggregation, phagocytosis, and release of bactericidal substances. Btk, and potentially other Tec family kinases, is a key component of the FcγRIIa intracellular signalling pathway. However, the role of these kinases in FcγRIIa signalling in response to bacteria is unknown. Moreover, the effects of iBtks on platelet responses to bacteria have not been evaluated. This study aims to investigate the role of Btk in platelet FcγRIIa signalling in response to bacterial agonists, and how this is affected by ibrutinib.

We show that ibrutinib and acalabrutinib inhibit healthy donor FcγRIIa-mediated platelet aggregation, alpha and dense granule release, in response to incubation with *Staphylococcus aureus* and *Escherichia coli*, and in response to FcγRIIa crosslinking with the anti-FcγRIIa monoclonal antibody IV.3. Moreover, we show platelets derived from ibrutinib-untreated CLL patients aggregate normally to bacteria in the presence of autologous plasma. However, platelets from ibrutinib-treated CLL patients have significantly inhibited aggregation, alpha granule release and bacteria scavenging. Platelet surface levels of FcγRIIa remained unchanged in both CLL groups, compared to healthy controls, however, levels of GPVI and αIIbβ3 were decreased in CLL samples regardless of ibrutinib therapy.

In both healthy control and ibrutinib-untreated CLL platelets, phosphorylation of Btk at tyrosine 223 (a marker of Btk activation) was detected in response to FcγRIIa agonists including *Staphylococcus aureus* and *Escherichia coli*. In contrast, Btk phosphorylation at Y223 in response to bacteria was absent in ibrutinib-treated CLL platelets, and in healthy controls platelets inhibited by ibrutinib and acalabrutinib *in vitro*. We also show a significant decrease in Tec phosphorylation in healthy control platelets treated with ibrutinib, and in ibrutinib-treated CLL platelets. To determine if Btk was required for platelet bacteria responses, X-linked

agammaglobulinaemia platelets, which lack a functional Btk, were exposed to bacteria and FcγRIIa-mediated platelet aggregation was observed, showing Btk to be unessential.

To conclude, our data shows that iBtks impair FcγRIIa-mediated platelet responses to bacteria both *in vitro* and at therapeutic conditions, and this impairment may be a result of both Btk and Tec inhibition, though potential off-target effects on other kinases cannot be dismissed. The effect of iBtks on platelet immune responses may possibly contribute to increase infections observed in CLL.

Publications

Posters:

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Abbreviations

α-granules	Alpha granules
ACD	Acid-Citrate-Dextrose
ADAM 17	A Disintegrin And Metalloprotease 17
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
BCR	B Cell Receptor
Bcl-2	B Cell Lymphoma 2
BH	Bruton's Tyrosine Kinase Homology
BSA	Bovine Serum Albumin
Btk	Bruton's Tyrosine Kinase
CFU	Colony Forming Units
CLEC2	C-type Lectin-Like Receptor 2
Clf	Clumping Factor
CLL	Chronic Lymphocytic Leukaemia
CLL-IPI	Chronic Lymphocytic Leukaemia International Prognostic Index
CRP	Collagen Related Peptide
DAG	Diacylglycerol
DNA	Deoxyribose Nucleic Acid
E. coli	*Escherichia Coli*
ELISA	Enzyme Linked Immunosorbent Assay
FISH	Fluorescent In Situ Hybridisation
FRC	Fludarabine Rituximab Cyclophosphamide Therapy
FSC	Forward Scatter
Gads	Growth Factor Receptor Bound Protein 2-Related Adapter Downstream of Shc
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GP	Glycoportein
GPCR	G Protein Coupled Receptor
GTP	Guanine Triphosphate

HIT	Heparin-Induced Thrombocytopenia
HRP	Horseradish Peroxidase
iBtk	Btk Inhibitor
Ig	Immunoglobulin
IGHV	Immunoglobulin Heavy-Chain Variable Region
IL	Interleukin
IRAK	Interleukin 1 Receptor Associated Kinase
IP_3	inositol-1,4,5-trisphosphate
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITIM	Immunoreceptor Tyrosine-Based Inhibitory Motif
Itk	Interleukin-2-Inducible T-cell Kinase
LAT	Linker for Activated T cells
LTA	Light Transmission Aggregometry
PAR	Protease-Activated Receptor
PH	Pleckstrin Homology
PI-3-K	Phosphoinositide 3-Kinase
PKC	Protein Kinase C
PLCγ2	Phospholipase C Gamma 2
PPR	Polyproline Region
RNA	Ribonucleic Acid
mAb	Monoclonal Antibody
MAPK	Mitogen Activated Protein Kinase
MCLL	Mutated Chronic Lymphocytic Leukaemia
MFI	Mean Fluorescent Intensities
MyD88	Myeloid Differentiation Factor 88
NET	Neutrophil Extracellular Trap
NFAT	Nuclear Factor of Activated T cells
NLRP3	NOD- LRR- and pyrin domain-containing protein 3
PAMP	Pathogen-Associated Molecular Patterns
PBS	Phosphate-Buffered Saline
PF4	Platelet Factor 4
PIP_3	of phosphatidylinositol (3,4,5)-trisphosphate
PPP	Platelet Poor Plasma

PRP	Platelet Rich Plasma
S. aureus	*Staphylococcus aureus*
S. gordonii	*Streptococcus gordonii*
S. sanguinis	*Streptococcus sanguinis*
S. oralis	*Streptococcus oralis*
SDS	Sodium Dodecyl Sulphate
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SH	Src Homology
SLL	Small Lymphocytic Lymphoma
SLP-76	SH2 Domain Containing Leukocyte Protein of 76kDa
SRR	Serine Rich Repeat
SpA	Surface Protein A
Src	Proto-Oncogene Tyrosine-Protein Kinase Sarcoma
SSC	Side Scatter
Syk	Spleen Tyrosine Kinase
TBS	Tris Buffered Saline
TH	Tec Homology
TLR	Toll-Like Receptor
TF	Tissue Factor
TK	Tyrosine Kinase
TP	Thromboxane Receptor
TPO	Thrombopoietin
TRAP6	Thrombin Receptor Activator Peptide 6
TRITC	Tetramethylrhodamine B Isothiocyanate
TXA_2	Thromboxane A2
UCLL	Unmutated Chronic Lymphocytic Leukaemia
VWF	von Willebrand Factor
XID	X-chromosome-linked immune-deficient
XLA	X-Linked Agammaglobulinaemia
ZAP-70	Zeta-Chain-Associated Protein Kinase 70

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Author's Declaration

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Chapter 1: Introduction

1.1 Introduction to CLL

1.1.1 Pathology of CLL

Chronic lymphocytic leukaemia (CLL) is the most common form of leukaemia in the Western World, with over 20,000 new cases a year in the United States (Siegel, Miller and Jemal, 2018). Mainly affecting the elderly, the survival of CLL can vary from months to years, however, the disease is incurable, with current treatments allowing for remission which most patients relapse from. Symptoms of CLL include frequent infections, weight loss, swollen glands, anaemia, and high temperature. Thrombocytopenia, neutropenia and anaemia are due to the infiltration of cancerous cells in the marrow (Hallek, Shanafelt and Eichhorst, 2018).

CLL is characterised by a clonal expansion of B cells in the blood and marrow, as well as secondary lymphoid tissue. These cells are mainly anergic, unresponsive in their immune function, hence why it was long believed that the disease was due to defects in apoptosis which resulted in an overaccumulation of the malignant cells. However, the leukaemia cells undergo high levels of turnover, found by isotopic labelling (Messmer *et al.*, 2005), at a rate which can be greater than 1% of the entire clone a day. This proliferation is thought to occur in pseudofollicles in the lymph nodes (Vandewoestyne *et al.*, 2011).

It is possible to define CLL into two classes by the immunoglobulin expressed on the B cells, encoded by either mutated (MCLL) or unmutated (UCLL) immunoglobulin heavy-chain variable region genes (IGHV). The mutational subgroups have different prognoses, with relatively unmutated IGHV genes being the most aggressive form of CLL (Hamblin *et al.*, 1999). This may be due to MCLL B cell receptor (BCR) high affinity binding to a restricted sets of antigenic epitopes which either cause anergy of high-affinity binding or are foreign antigens, so binding occurs infrequently. This results in the clonal expansion of the CLL clone, ensuring the B cell remains stable and the expansion rate is slow (Chiorazzi and Ferrarini, 2003). In UCLL, however, the BCR has low affinity reactions to many antigens (Bröker *et al.*, 1988) (Binder *et al.*, 2010) leading to increased activation through BCR-mediated signalling. The mutational status

of the IGHV genes suggests UCLL B cells may arise from a naïve B cell, while MCLL B cells arise from post-germinal centre B cells as they also have mutated IGHV genes. Specific antigens which interact with the B cell receptor of CLL cells have been identified which may influence the disease to either MCLL or UCLL (Hervé *et al.*, 2005) (Myhrinder *et al.*, 2008) (Hoogeboom *et al.*, 2013). Both types are shown to overexpress activation markers CD25, CD69 and CD23 and CD71, and also under express markers which are downregulated by activation such as CD22, CD79b and FcγRIIb (Chiorazzi and Ferrarini, 2003). Cells in CLL have a characteristic immunophenotype which differentiates them from other lymphoproliferative diseases, which includes CD5, CD19 and CD23 expression.

1.1.2 Diagnostic and prognostics in CLL

For a diagnosis of CLL, an absolute lymphocyte count of at least 5000 cells/mm^3 is required for a minimum of three months (Hallek *et al.*, 2018), and is usually discovered during routine laboratory tests as most patients are asymptomatic at diagnosis. In the blood smear, as well as smudge cells commonly being seen, the lymphocytes have a narrow cytoplasmic border within which there is aggregated chromatin and a dense nucleus with no nucleoli. Small lymphocytic lymphoma (SLL), occurring in 5% of CLL cases, is characterised by CLL cells confined to the lymph nodes and other tissues, but not yet present in the blood or bone marrow. SLL should be diagnosed with a lymph node biopsy due to the lower levels of B cells in the blood.

Upon diagnosis, prognostic tests are available. Flow cytometry is then used to measure immunoglobulin light chain restriction to determine if the cells are clonal. A marrow biopsy and aspirate can indicate tumour burden by determining the extent and the either diffuse or non-diffuse pattern of marrow infiltration. A non-diffuse pattern of bone marrow involvement is associated with longer survival of the patient (Montserrat and Rozman, 1987). Serum markers can also be used as a prognostic test, such as serum thymidine kinase (Hallek *et al.*, 1999), β2-microglobulin (Amaya-Chanaga and Rassenti, 2016) and CD23 (Knauf *et al.*, 1997).

Cytogenetic lesions can be identified in CLL by fluorescent in situ hybridisation (FISH) in over 80% of cases (Baliakas *et al.*, 2014) and four of these can be used as prognostic

markers: trisomy chromosome 12 and deletions in chromosomes 11q, 13q and 17p. The pathophysiology to trisomy 12 CLL is relatively unknown, however, patients with the trisomy have higher levels of *NOTCH1* which can act as an oncogene, higher proliferation rate of B cells, higher rates of secondary cancers, and higher rates of thrombocytopenia (Abruzzo *et al.*, 2018). Deletions in 11q chromosomes are associated with rapid disease progression and increased lymph node involvement, and loss of ataxia telangiectasia mutated gene is common. The ataxia telangiectasia mutated gene is involved in deoxyribose nucleic acid (DNA) damage response, and in the case of biallelic loss, this can lead to chemoresistance (Stankovic and Skowronska, 2014) (Döhner *et al.*, 1997). Patients with deletions in chromosome 13q have a better prognosis than patients with 17p chromosome deletions which will hence have a dysregulated p53. Mutations in the *TP53* gene are also associated with an aggressive form of the disease (Dicker *et al.*, 2009). Patients with either or both 17p chromosome mutations and *TP53* mutations should be treated with nonchemotherapeutic agents, such as Bruton's tyrosine kinase inhibitors (e.g. ibrutinib) or phosphatidylinositol 3-kinase inhibitors (e.g. idelalisib), instead of chemoimmunotherapy to which they respond poorly (Fischer *et al.*, 2012) (Hallek *et al.*, 2008) (Stilgenbauer *et al.*, 2014).

There are two main clinical staging systems used for CLL patients: the Binet (Binet, Vaugier, *et al.*, 1977) staging system and the Rai (Rai *et al.*, 1975) staging system, the difference in which is shown in Table 1.1.

Table 1.1. Comparison of Binet and Rai Staging of CLL

Staging System	Clinical Features	Mean Survival/Years (Gribben, 2010)
Binet Staging System		
A	< 3 areas of lymphadenopathy	12
B	> 3 areas of lymphadenopathy	7
C	Haemoglobin < 100g/L Platelets < $100x10^9$/L	<4
Rai Staging System		
0	Lymphocytosis of blood and bone marrow	>10
I	Lymphadenopathy	7
II	Splenomegaly Hepatomegaly	7
III	Haemoglobin <11g/dL	2
IV	Platelets <$100x10^9$/L	2

More recently the CLL international prognostic index (CLL-IPI) has been developed to be used as a clinical staging system for CLL. It incorporates prognostic biomarkers such as the *TP53* gene, β2-microglobulin and IGHV mutations, as well as biological factors such as age and clinical stage. It aimed to prevent redundant tests for poor clinical outcomes, such as CD38 and ZAP-70 (zeta-chain-associated protein kinase 70) (International, 2016).

In a study approved by the Mayo Clinic Institutional Review Board, 1274 patients diagnosed over a 19-year span were monitored, in which the cause of the 286 deaths which occurred in this time frame were determined to be due to disease progression in 34.6%, infection in 5.6% and second malignancy in 16.4% (Wang *et al.*, 2021). In another study, of 1174 patients over 12 years 224 died, of which 74% was due to disease progression and 14% was due to infection (Strati *et al.*, 2015). Another study of 105 patients found that of 55 deaths over a 25-year period, 44% was due to infection (Robertson, 1990).

1.1.3 Management of CLL

Historically, alkylating agents and purine analogues were used for the treatment of CLL. Alkylating agents damage the cancer cells by creating interstrand-crosslinks within the DNA, the purine analogues then prevent the repair of the damaged DNA, preventing replication of the cancer cell. One such treatment is the nitrogen mustard alkylating agent chlorambucil (Markman, 2003). On top of DNA damage, chlorambucil has also been proposed to alkylate other cellular targets in order to produce the apoptotic effect seen in CLL cells (Begleiter *et al.*, 1996). However, there can be resistance to this drug. In around 15% of CLL patients, there is a mutation or a deletion in *TP53* which correlates to a poor response to chlorambucil, which also indicates that the drugs apoptotic activities are *TP53* dependant. Another pathway to resistance can be elevated levels of activity of glutathione S-transferase, which mediate detoxification of chlorambucil.

Chlorambucil was the major form of treatment of CLL until the 1980's when fludarabine monophosphate, a purine analogue, was found to be an effective treatment. Due to the negative charge on the drug, it is unable to enter cells, so relies on dephosphorylation to produce the antimetabolite F-ara-A. Fludarabine then enters the cell via facilitated transport (Barrueco *et al.*, 1987), where deoxycytidine kinase phosphorylates it to F-ara-ATP. F-ara-ATP inhibits DNA synthesis in different ways, one of which is competing with the deoxynucleotides for incorporation into the DNA, usually at the 3' end, and hence inhibiting polymerisation by preventing DNA ligase I from being able to join it to adjacent strands. This mechanism can be accentuated by the inhibitory effects of F-ara-ATP on ribonucleotide reductase, which decrease cellular levels of the deoxynucleotides (Parker *et al.*, 1988). Another way fludarabine exerts effects is by the inhibition of lagging strand polymerisation by inhibiting DNA primase. DNA primase which would usually synthesise the ribonucleic acid (RNA) primer for the lagging strand (Catapano, Perrino and Fernandes, 1993), and hence inhibition would prevent lagging strand formation.

Fludarabine has superior response rates and longer durations of relapse-free survival compared to chlorambucil (Rai *et al.*, 2000), however, when both drugs were compared in the elderly, no significant difference in progression-free survival was seen

(Eichhorst *et al.*, 2009). Fludarabine may not be a treatment option for certain patients, called fludarabine-ineligible. This ineligibility may be due to factors such as age and frailty, as well as compromised kidney function as this is the mode of fludarabine elimination. Another predictor of fludarabine toxicity is extensive bone marrow infiltration resulting in pancytopenia.

Both chlorambucil and fludarabine can be combined with other therapeutic agents to increase efficacy. One such agent is rituximab, a chimeric anti-CD20 antibody engineered as a human/mouse immunoglobulin (Ig) G1-κ monoclonal antibody. CD20, expressed on the surface of all B cells starting in the pro-B stage and increasing in levels until maturity, is a glycosylated phosphoprotein which acts as a calcium channel that interacts with the B cell immunoglobulin receptor complex. CD20 levels are decreased in CLL (Almasri *et al.*, 1992), which could act as a biomarker for the disease. The use of rituximab can result in death of B-cells by apoptosis in both a caspase-dependent and independent manner. However, cell death can also occur by the recruitment of other immune cells such as natural killer cells which recognise rituximab through FcγRIII, or macrophages through FcγRs, both of which lead to antibody-dependent cytotoxicity and antibody-dependent phagocytosis respectively. Rituximab can also trigger the complement cascade with the end result of complement-dependent cytotoxicity (Pierpont, Limper and Richards, 2018).

Several randomised studies demonstrated that rituximab in combination with other chemotherapeutic agents increased both the overall response rate and the overall survival. The rationale for combination of fludarabine with rituximab was due to rituximab's chemosensitisation and lysis of tumour cells thereby increasing the efficacy of fludarabine. Rituximab has also been seen to increase fludarabine's cytotoxicity (Demidem *et al.*, 1997) (Alas, Bonavida and Emmanouilides, 2000). Fludarabine had previously been combined with the alkylating agent cyclophosphamide due to the synergism between the two agents which increased apoptosis to greater levels than when both were used as treatment alone (Yamauchi *et al.*, 2001) (O'Brien *et al.*, 2001), as such rituximab has since been combined with both of these. The combination of these drugs has been proven to be effective in many trials (Tam *et al.*, 2008), and the addition of rituximab to fludarabine and cyclophosphamide therapy (FRC) also proves

more effective than fludarabine and cyclophosphamide therapy alone (Keating *et al.*, 2005). The effects of this therapy are greater for those patients with MCLL, who have prolonged progression free survival and increased rates of negative minimal residual disease than UCLL in response to FCR (Thompson *et al.*, 2016). FCR has also been shown to be an effective treatment for relapsed patients, more so than other available treatments (Badoux *et al.*, 2011).

Whilst being first-line treatment for fit patients, FCR is not an effective treatment for unfit or elderly patients (Eichhorst, Goede and Hallek, 2009), hence rituximab is combined with chlorambucil instead. Chlorambucil-rituximab is preferable to chlorambucil monotherapy due to the improvement in survival rates (Foà *et al.*, 2014) (Hillmen, Gribben, *et al.*, 2014) (Laurenti *et al.*, 2013). However, chlorambucil-rituximab therapy, though having decreased toxicity also has reduced efficacy (Hillmen, Gribben, *et al.*, 2014).

Newer targeted therapies directed against specific proteins have been developed. One such protein is B cell lymphoma 2 (Bcl-2), which plays a role in a specific type of apoptosis, namely the intrinsic pathway. This pathway is activated by different signals including lack of growth factors, radiation or cellular stress which results in the release of proteins, such as cytochrome c from the mitochondrial intermembrane space via mitochondrial outer membrane permeabilisation. Released cytochrome c in the cytosol interacts with the adaptor protein apoptotic protease activating factor 1 and the inactive caspase procaspase 9. This creates the apoptosome. Caspase 9 is then activated which triggers the caspase activation cascade which results in apoptosis. Bcl-2 prevents the release of cytochrome c.

The RNA and protein levels of Bcl-2 have been reported to be elevated in CLL (McCarthy *et al.*, 2008), especially on the cell surface (McCarthy *et al.*, 2008). Venetoclax is a Bcl-2 inhibitor that binds to Bcl-2 causing it to signal the apoptotic cascade for cell death. In the M13-982 trial, it was demonstrated that this drug was therapeutically effective in patients with TP53 mutations and in patients which had previously received at least two prior treatments. In 11% of patients this prior treatment was a B-cell receptor signalling inhibitor such as ibrutinib or idelalisib, the

effects of which have also been evaluated in other studies (Coutre *et al.*, 2018) (Jones *et al.*, 2018) (Jones *et al.*, 2015). Venetoclax is effective as a monotherapy (Stilgenbauer *et al.*, 2016) (Stilgenbauer *et al.*, 2018) (Roberts *et al.*, 2017), and more recently in combination with other drugs such as rituximab or ibrutinib (Seymour *et al.*, 2018) (Seymour *et al.*, 2017).

As well as p53 and Bcl-2, another important protein involved in the pathogenicity of CLL is the BCR. The BCR is expressed on mature B cells as two isotypes: IgD and IgM. The BCR is composed of membrane immunoglobulin which consists of two light chains and two heavy chains. The heavy chains consist of either consists of 4 or 5 immunoglobulin domains in the cases of IgD and IgM respectively. The BRC also contains a hinge, and a three amino acid intracellular domain (K, V, K). The immunoglobulin molecule lacks a signalling motif and is associated with a Igα/Igβ heterodimer (Schamel and Reth, 2000) whose cytoplasmic domain contains a conserved sequence of amino acids (YxxL/I), called immunoreceptor tyrosine-based motif (ITAM), two of which sequences are usually separated by 7 to 12 amino acids. A proposed mechanism for the activation of the BCR is the dissociation activation model (Yang and Reth, 2010). In this model, the majority of resting BCRs are autoinhibited closed oligomers, whilst the remaining few remain monomeric and open, possibly to provide a tonic signal for survival (Monroe, 2006). Upon the binding of a polyvalent antigen, the equilibrium of the BCRs shifts towards the monomeric active state, leading to exposed ITAMs which are then phosphorylated by proto-oncogene tyrosine-protein kinase Sarcoma (Src)-family kinases Lyn, Fyn and Blk. The phosphorylated ITAM can then act as a docking site for the SH2 domain-containing spleen tyrosine kinase (Syk) protein, leading to Syk activation. The activated Syk triggers a signalling cascade which includes the activation of Bruton's tyrosine kinase (Btk), phosphoinositide 3-kinase (PI3-K), nuclear factor of activated T cells (NFAT) and mitogen activated protein kinase (MAPK) pathways. This promotes cell proliferation and survival (Woyach, Johnson and Byrd, 2012).

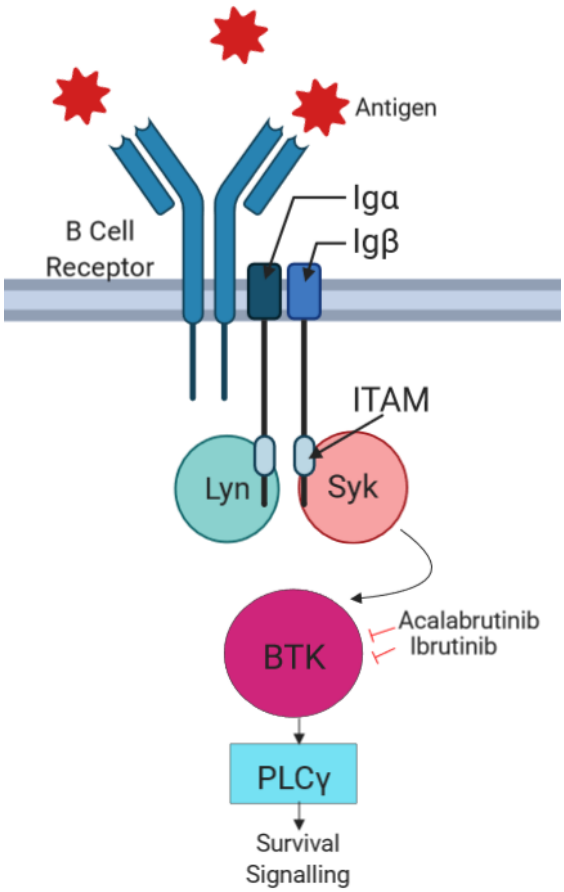
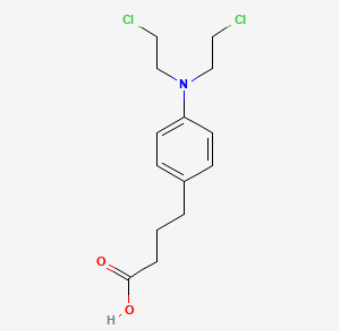
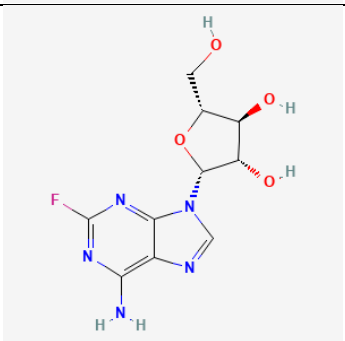
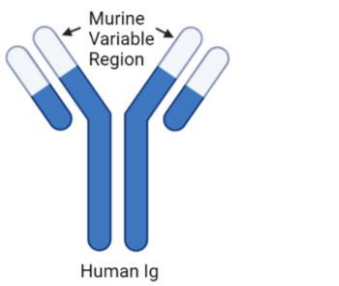
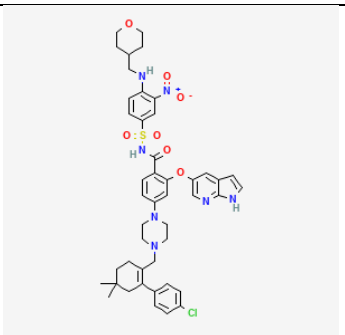


Figure 1.1. B cell signalling through BCR. Adapted from (Burger and Wiestner, 2018).

There is a low surface expression of the BCR in CLL compared to the healthy population, possibly due to folding and glycosylation impairments (Vuillier *et al.*, 2005). Despite the lower expression levels, the BCR can still be targeted in the treatment of CLL with drugs designed to target the BCR signalling pathway. These drugs are Btk inhibitors and include first generation ibrutinib which binds to Btk. This prevents downstream signalling of the BCR and hence prevents B cell proliferation. Inhibitors of Btk with be discussed further in section 1.5.6.

Table 1.2. Summary of non Btk inhibiting CLL treatments. Chemical structures taken from PubChem.

Drug	Structure and Pharmacology	Mechanism of Action
Chlorambucil		Alkylating agent
Fludarabine		Purine Analogue
Rituximab		Monoclonal antibody for CD20
Venetoclax		Bcl-2 inhibitor

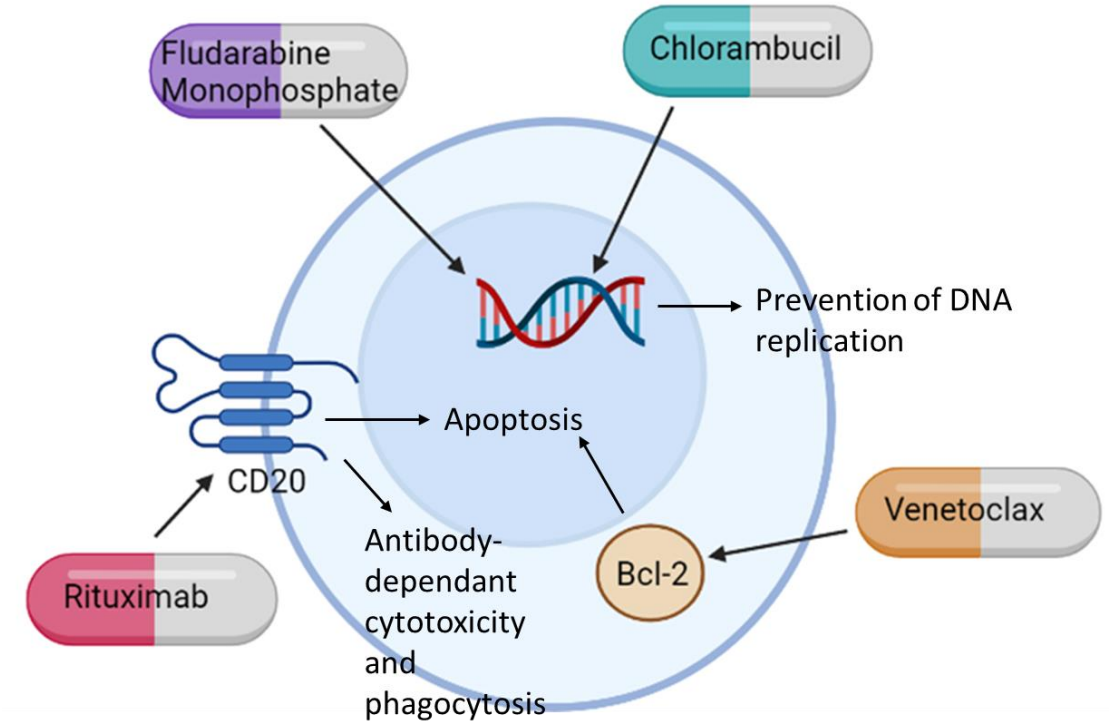


Figure 1.2. CLL treatments and their targets in B cells.

1.2 Introduction to Platelets

Platelets are small, anucleate cell fragments with nearly one trillion found in an adult humans' circulation, with normal levels of 150-450x10^9/l. Found only in mammals, platelets are thought to have evolved from haemocytes of marine invertebrates. In the latter, haemocytes can prevent vessel leakage via aggregation, as well as carry out phagocytosis of pathogens. In non-mammalian vertebrates, the platelet equivalent is the nucleated thrombocyte (Stosik, Tokarz-Deptuła and Deptuła, 2019).

Platelets have some unique receptors as well as some receptors found in other cells, an organised cytoskeleton, and secretory granules. Platelets key role is the response to blood vessel injury by the formation of a haemostatic plug, as well as in inflammatory haemostasis by preventing vasculature leakage from the site of inflammation (Periayah, Halim and Saad, 2017). Quantitative and qualitative platelet defects result in thrombocytopenia or impaired aggregatory responses and result in bleeding. For

example, Glanzmann thrombasthenia is caused by a deficiency or defect in αIIbβ3 which results in aggregation and platelet-platelet interaction abnormalities (Kirchmaier and Pillitteri, 2010). Other defects cause thrombocytopenia's as seen in x-linked thrombocytopenia which is an inherited clotting disorder that is also associated with infections (Albert *et al.*, 2010).

1.2.1 Platelet production and structure

Platelets are formed from progenitor cells called megakaryocytes. Megakaryopoiesis occurs in the bone marrow and possibly in the lung (Lefrançais *et al.*, 2017). In the bone marrow, the mature megakaryocyte rearranges its cytoplasm to form pseudopodia, the beginning of a proplatelet. This pseudopodium elongates into a teardrop shape, with a loop of microtubules at the distal tip. This proplatelet then further branches from which more pseudopodia extend, resulting in constrictions along the processes, forming swellings which are thought to be due to the delivery of platelet material to the tips of the branches. Finally, the megakaryocyte retracts causing the release of the proplatelet. The proplatelet then undergoes further fragmentation to form platelets (Italiano *et al.*, 1999). A megakaryocyte can produce around 2000-3000 platelets in its lifespan. A platelet will then survive in circulation for up to 10 days. During this time, the platelet is reduced in size (Karpatkin, 1969). At the end of the platelets life, they are transported to the liver where they are cleared by the reticuloendothelial system (Kaplan and Saba, 1978).

Thrombopoietin (TPO), produced in the liver, promotes the production of platelets (Kaushansky, 2005). Platelets express TPO receptors to which TPO binds to be internalised and degraded (Varghese *et al.*, 2017). If non-bound TPO exceeds the level of TPO receptors on the platelet, then remaining TPO binds to megakaryocytes to contribute to platelet production via the activation of the Janus kinase-signal transducer and activator of transcription pathway, which serves to cause the maturation of the megakaryocyte and well as increase the number of platelets. This results in an increase in megakaryocyte production (Kuter, 2013). Hence the effects of TPO are subject to negative regulation by platelet mass: the more platelets the less TPO goes to megakaryocytes the less platelets are produced; and vice versa.

Platelets have a smooth plasma membrane associated with a glycocalyx, which acts as a dynamic structure, being the first contact with the surrounding environment. The glycocalyx is associated with glycoproteins (Alphonsus and Rodseth, 2014). The glycocalyx associates with a lipid bilayer. When platelets spread, extra membrane needs to be supplied from either the open canalicular system or from folds in the membrane, due to the lipid bilayer being unstretchable (Behnke, 1970). It is the lipid bilayer which expresses tissue factor (TF) on activated platelets and initiates coagulation (Butenas, Orfeo and Mann, 2009). Beneath the lipid bilayer there is a submembrane containing thick actin filaments which mediate shape change. Furthermore, these actin filaments and microtubules are also involved in secretion and cellular transport (White, 1969).

Within the platelet there is the sol-gel zone, a viscous matrix of microfilaments and organised microtubules (Geraldo *et al.*, 2014). The organelles of the platelet are suspended separately from one another while actin microfilaments form the actin cytoskeleton. The coils of the microtubule constrict upon platelet activation, resulting in the movement of α and dense granules to the centre of the platelet resulting in their secretion through the open canalicular system (Escolar, Krumwiede and White, 1986). Thought to maintain the discoid shape of the platelet (White and Krivit, 1967), microtubules are close to the cell wall to support the contractile cytoskeleton of the membrane (Behnke, 1965). The sol-gel zone also contains glycogen, and smooth and clathrin-coated vesicles (Gremmel, Frelinger and Michelson, 2016).

Intracellular platelet organelles include mitochondria and glycosomes. They also contain the Golgi apparatus, rough endoplasmic reticulum and the open canalicular system. The open canalicular system is formed from the outer membrane and extends towards the centre of the platelet. The channels formed allow for granular content secretion upon platelet activation, and are evaginated during platelet spreading to provide the platelet with greater surface area (Selvadurai and Hamilton, 2018). On top of this, the open canalicular system can be used to transport substances into and out of the platelet (Selvadurai and Hamilton, 2018).

1.2.2 α-granules

Each platelet contains fifty to eighty α-granules (King and Reed, 2002), which are 200-500nm in diameter. The α-granule contains plasma components such as fibrinogen, as well other proteins such as von Willebrand factor (VWF). Megakaryocyte-synthesised proteins are also found within α-granules, such as P-selectin, often used as a marker for platelet activation, and thrombospondin (Blair and Flaumenhaft, 2009).
α-granules contain a vast array of chemokines, such as: CXCL4 (platelet factor 4 (PF4)), CXCL7, CCL5, CXCL12, CCL2 and CCL3. Together, CCL5 and PF4 can cause monocyte and endothelial cell adhesion to platelets. In addition, PF4 causes adhesion of neutrophils. PF4 also has effects on monocytes, such as activation, and differentiation to foam cells and macrophages. In addition to being a chemoattractant for neutrophils and fibroblasts, PF4 neutralises heparin-like molecules on the endothelial surface of blood vessels to promote coagulation (Bikfalvi, 2004). CLCX7 is another platelet chemokine, which can be proteolytically cleaved into four different chemokines: neutrophil activating peptide 2, penicillin binding proteins, β-TG and connective tissue activating peptide III. Neutrophil activating peptide 2 is the only produced chemokine from this cleavage to possess a large activity. CXCL7 is seen to cause endothelial cell adhesion of neutrophils, as well as neutrophil chemotaxis (Schenk *et al.*, 2002).

When platelets are activated, the α-granules release their contents; the soluble contents are released extracellularly, whereas the α-granule membrane-bound contents become expressed on the platelet membrane surface. This can include specific proteins that are only expressed upon activation, such as P-selectin, or proteins which are already expressed on the platelet surface, such as αIIbβ3 (Blair and Flaumenhaft, 2009).

1.2.3 Dense granules

Dense granules in platelets are expressed at a much lower level than α-granules, with three to eight per platelet, and are also much smaller, being described as electron-opaque spheres. Dense granules contain assorted compounds like bioactive amines (e.g. histamine and serotonin), cations (e.g. calcium and potassium), and a high concentration of adenine nucleotide (e.g. adenosine triphosphate (ATP) and adenosine diphosphate (ADP)) (Chen, Yuan and Li, 2018). They also contain glutamate which is

seen to cause T-cell migration in the periphery (Ganor *et al.*, 2003), as well as serotonin which can cause the differentiation of monocytes into dendritic cells (Katoh *et al.*, 2006) on top of naïve T-cell activation (León-Ponte, Ahern and O'Connell, 2007). Another constituent of dense granules is polyphosphates which increase the expression of endothelial adhesion molecules and NFκB (Ruiz *et al.*, 2004).

1.3 Platelet Receptors

Platelets express many surface receptors, however, vital ones for adhesion and aggregation in haemostasis are glycoprotein (GP)VI, GPIb-IX-V, and αIIbβ3 (Moroi *et al.*, 1997) (Nieswandt and Watson, 2003) (Huang *et al.*, 2019). In haemostasis, agonists signal through two types of receptors: tyrosine-kinase linked receptors and G-protein coupled receptors. Both types of receptors converge on the common pathway of αIIbβ3 inside-out signalling. There are three crucial positive feedback mechanisms for platelet activation: thromboxane A_2 (TXA_2) which is produced de novo, ADP which is released from dense granules, and αIIbβ3 outside-in signalling. The basic steps in thrombus formation involves platelet tethering and firm adhesion to collagen and VWF at the subendothelium by platelet receptors GPVI (collagen), GPIb-IX-V (VWF), and αIIbβ3 (VWF). This causes the positive feedback through secondary mediators such as ADP, which serves to further activate platelets through G-protein coupled receptors (GPCR) which in turn activates inside-out signalling of αIIbβ3. αIIbβ3 undergoes a conformational change to the active conformation to bind fibrinogen which allows for platelet-platelet aggregation. Thrombus formation is self-mediated with negative feedback mechanisms driven by immunoreceptor tyrosine-based inhibitory motif receptors, desensitisation of receptors and negative regulation of αIIbβ3 by endothelial cell-selective adhesion molecule (Bye, Unsworth and Gibbins, 2016).

1.3.1 Adhesion receptors

GPIb-IX-V is a prominent receptor involved in platelet adhesion. The receptor is a complex of GPIbα, GPIX, GPV, and two GPIbβ subunits (Andrews and Berndt, 2013). GPIbα spans the membrane, with an N-terminal ligand-binding domain consisting of seven tandem leucine-rich repeats. There is double the expression of GPIbα and GPIX

compared to GPV, at 25000 copies to 12500 copies respectively (Kauskot and Hoylaerts, 2012). The main ligand of GPIb-IX-V is VWF. Collagen can also bind to GPIb-IX-V indirectly, via VWF (Nieswandt and Watson, 2003). Such binding is seen in platelet adhesion to injured vessel endothelial cells as well as to subendothelial structures. When VWF is absent at times of high shear, GPIb-IX-V can bind to thrombospondin to mediate adhesion (Jurk *et al.*, 2003). Thrombospondin is found on activated platelets (Lawler, Slayter and Coligan, 1978) but can also be secreted from other cells such as monocytes, endothelial cells and macrophages (Gutierrez, Lopez-Dee and Pidcock, 2011). P-selectin is another ligand of GPIb-IX-V, allowing for platelet-platelet binding, as well as platelet-endothelial cell binding (Romo *et al.*, 1999).

αIIbβ3 is an integrin expressed on the surface of platelets at about 80000 to 100000 copies per cell (Wagner *et al.*, 1996), which accounts for 17% of the membrane protein mass in platelets (Phillips *et al.*, 1988). αIIbβ3 is also expressed in the membranes of α-granules, allowing for increased expression after platelet activation (Joo, 2012). αIIbβ3 mediates thrombus formation, adhesion, and aggregation in response to binding to its ligands which includes VWF and fibrinogen.

αIIbβ3 is normally held in a 'resting' low affinity conformation. Upon agonist stimulation of other receptors, downstream signalling pathways converge to the cytoplasmic tail of αIIbβ3 causing what is known as inside-out activation of αIIbβ3, by which the integrin changes to a high-affinity conformation allowing it to bind VWF and fibrinogen, resulting in adhesion. Outside-in signalling through αIIbβ3 is achieved through the extracellular domains of the receptor binding to multimeric ligands. The result of this is platelet spreading, adhesion, thrombus formation, aggregation, degranulation and clot retraction (Joo, 2012).

GPVI is an adhesion receptor which will be detailed later in this chapter.

1.3.2 G protein coupled receptors

GPCRs form a family of proteins with seven transmembrane domains which signal through heterotrimeric G proteins consisting of the subunits α, β and γ. The main agonist of GPCRs in platelets include ADP, thrombin, and thromboxane. ADP binds to

the P2Y12 and P2Y1 receptors, thrombin binds to protease-activated receptors (PAR) 1 and PAR4, and thromboxane binds to thromboxane receptor (TP) (Figure 1.3). The α-subunit of the GPCR is guanine diphosphate (GDP)-bound when inactive, but guanine triphosphate (GTP)-bound when activated. This allows the α-subunit to dissociate from the receptor and β/γ-complex. The α-subunit can then interact with downstream signalling targets, as does the β/γ-complex (Offermanns, 2006).

G proteins are divided into subfamilies, each with different downstream targets. Gq mediates signals from ADP and thromboxane stimulation. Gq is involved in shape-change induced by ADP, for granule secretion, aggregation and integrin activation, mainly from activating PLCβ (Offermanns *et al.*, 1997). Thrombin responses are also reliant on Gq. Gi couples with responses from thromboxane and ADP stimulation. Gi acts through inhibiting cAMP production to alleviate platelet inhibition, as well as activate though phosphoinositide 3-kinase (PI3K), to result in granule secretion and thromboxane production (Offermanns, 2006).

Gα13 is involved in serotonin and thromboxane signalling. Gα13 binds to guanine nucleotide exchange factors (GEFs) to cause their activation. GEFs activated include RhoA GEF, converting RhoA into its active GTP bound conformation, to result in the activation of Rho kinase. This results in the inhibition by phosphorylation of myosin light-chain phosphatase, which phosphorylates myosin light-chain causing contraction. The consequence of this is platelet shape change and granule secretion (Offermanns, 2006).

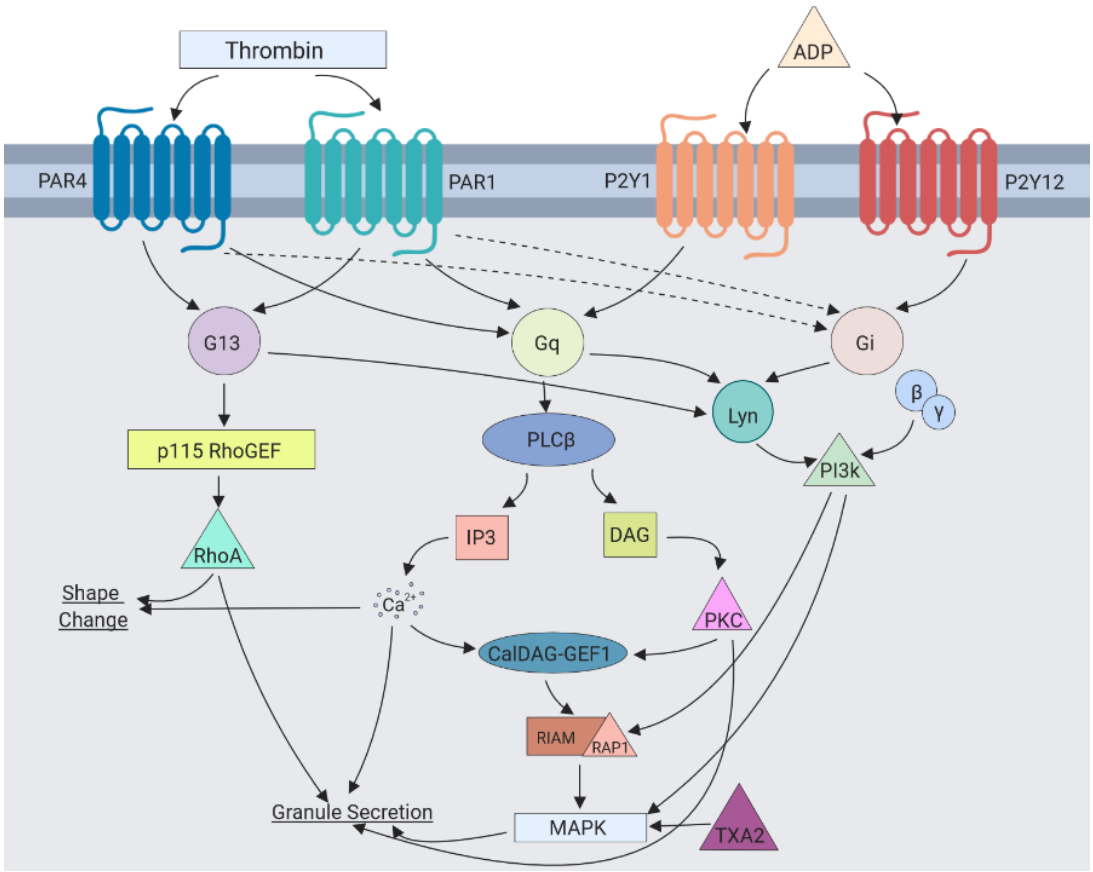


Figure 1.3. Platelet signalling through GPCR. Adapted from (Li *et al.*, 2010) and (Offermanns, 2006).

1.3.3 Toll-like receptors

Toll-like receptors (TLRs) are type 1 transmembrane proteins originally found on immune cells and are involved in immune responses and binding to pathogen-associated molecular patterns (PAMPs) (Armant and Fenton, 2002). PAMPs interact with TLR leucine-rich ectodomain. TLRs contain a transmembrane domain and a cytoplasmic domain called Toll-interleukin-1-receptor domain. Human platelets express TLRs 2, 4 and 9 (Cognasse *et al.*, 2005) with TLRs 2 and 4 expressed on the platelet surface, while TLR9 is expressed on endosomes. Each TLR has a different signalling outcome upon activation.

TLR4 is highly expressed by platelets (Berthet *et al.*, 2010). TLR4s main agonist is lipopolysaccharide (LPS), and activation of TLR4 by LPS results in an increase of TF expression (Rondina *et al.*, 2011). Signalling of TLR in platelets is less clear than in nucleated cells. TLR 2 and 4 in platelets utilises the myeloid differentiation factor 88 (MyD88) pathway (Zhang *et al.*, 2009), in which MyD88 interacts with interleukin (IL)-1

receptor associated kinase (IRAK) 4, and further interacts with IRAKs 1 and 2. Which in nucleated cells results in the phosphorylation of IRAKs which dissociate from MyD88 and interact with tumour receptor-associated factor 6, which activates transforming growth factor beta activated kinase and transforming growth factor beta activated kinase binding protein 2/3 to activate MAPK. Activation of MAPK results in the translocation to the nucleus of NFκB and activator protein 1 (Zheng *et al.*, 2020).

1.3.4 GPVI

1.3.4.1 Structure of GPVI

GPVI signalling pathway in platelets is involved in collagen-induced platelet activation (Figure 1.4). GPVI is a 319-residue long glycoprotein and a member of the immunoglobulin superfamily, and as such the extracellular region contains two immunoglobulin-like domains. Also, in the extracellular region there is a mucin-like domain which is rich in serine and threonine. GPVI is non-covalently associated with Fc receptor γ chain (FcRγ) (Tsuji *et al.*, 1997) through an arginine residue in GPVI's transmembrane domain forming a salt bridge with an aspartic residue in FcRγ's transmembrane domain (Berlanga *et al.*, 2002). Patients lacking FcγR also lack GPVI (Marjoram *et al.*, 2014). In the cytoplasmic domain of GPVI, close to the transmembrane domain, is a region rich in basic amino acids where calmodulin binds. There is a proline-rich region close to the middle of the cytoplasmic domain of GPVI, which binds selectively to Src family tyrosine kinases Lyn and Fyn via their Src homology 3 (SH3) domains (Moroi and Jung, 2004). Although GPVI is a major receptor for collagen, it has many other ligands such as fibrinogen and adiponectin (Haining *et al.*, 2019).

1.3.4.2 Signalling of GPVI

Collagen binding to GPVI induces the crosslinking of GPVI. This allows the FcRγ cytoplasmic ITAM domain to be phosphorylated on a tyrosine by Src-family kinases that are bound to the cytoplasmic proline-rich domain of GPVI (Ezumi *et al.*, 1998). These phosphorylated tyrosine residues act as a docking site for the SH2 domain containing tyrosine kinase Syk, allowing the phosphorylation of targets such as SH2 domain-containing leukocyte phosphoprotein of 76kDa (SLP-76), and linker for activated T cells (LAT). This process generates the signalling complex consisting of the

transmembrane adaptor LAT, cytosolic adaptors growth factor receptor bound protein 2-related adapter downstream of Shc (Gads) and LAT, phospholipase C gamma 2 (PLCγ2) and Btk which leads to the activation of PLCγ2 (Nieswandt and Watson, 2003). Activated PLCγ2 results in the activation of the protein kinase C (PKC) and the MAPK pathways which results in granule secretion, TXA_2 synthesis, and integrin activation. Deletion of the adaptor protein SH2 domain containing leukocyte protein of 76kDa (SLP-76) from the signalosome leads to almost complete loss of signalling from GPVI (Bezman *et al.*, 2008), however, the loss of LAT strongly impairs GPVI signalling, and the loss of Gads is better tolerated with only mild impairment of GPVI signalling (Hughes *et al.*, 2008).

Btk and Tec kinase have a regulatory role in GPVI signalling. In humans, the role of Btk has been studied using platelets from X-linked agammaglobulinaemia (XLA) patients, a condition in which Btk is not functional. In platelets derived from XLA patients signalling via GPVI in response to collagen and CRP is diminished in terms of PLCγ2 phosphorylation (Quek, Bolen and Watson, 1998). Patients with XLA lack a functional Btk and as a result have diminished platelet aggregation, Ca^{2+} mobilisation, and granule secretion responses to GPVI stimulation (Quek, Bolen and Watson, 1998). The fact that such responses are not completely extirpated is due the ability of Tec to partially substitute for Btk activity. Mice which are deficient in both Btk and Tec are unable to undergo such responses to GPVI ligation, however they can aggregate as normal in response to ADP (Atkinson, Ellmeier and Watson, 2003a). Ibrutinib also causes the same effects as seen in Tec and Btk deficient mice, due to ibrutinib inhibiting both Btk and Tec, while ADP still causes normal aggregation due to the lack of inhibition of the GCPRs P2Y1 and P2Y12 (Kamel *et al.*, 2015). This effect of ibrutinib on GPVI signalling has been demonstrated in patients treated with this drug who show a decrease in collagen-mediated platelet aggregation, which, after a week of not taking ibrutinib, the effect was reversed (Levade *et al.*, 2014). However, patients with XLA do not show the same bleeding phenotype as patients on ibrutinib (Pal Singh, Dammeijer and Hendriks, 2018) indicating possible off-target effects of ibrutinib.

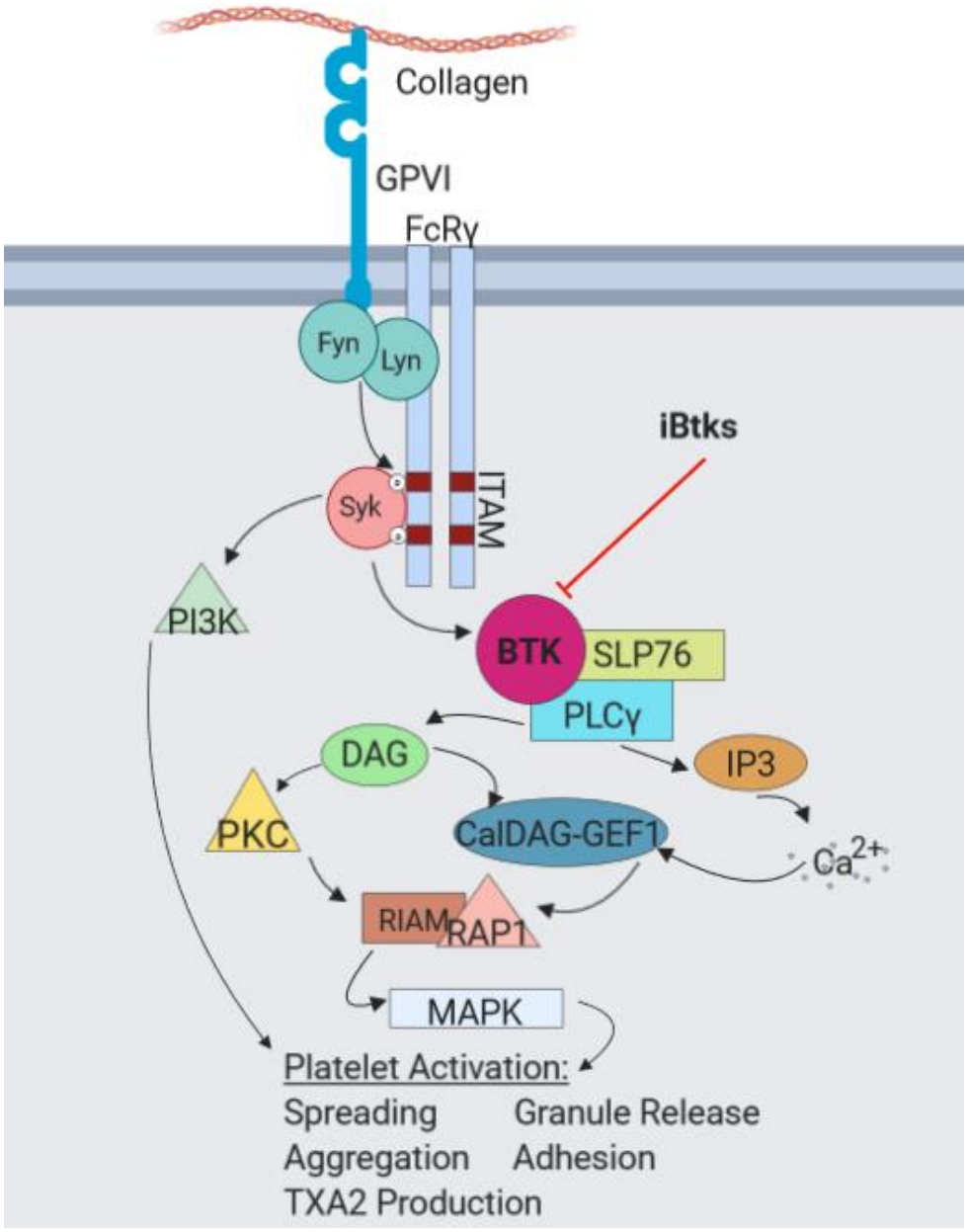


Figure 1.4. Platelet signalling through GPVI. Adapted from (Li et al., 2010).

1.3.5 CLEC2

1.3.5.1 Structure of CLEC2

C-type lectin like receptor 2 (CLEC2) has a minimal role in haemostasis, but has recently been found to have a role in thrombo-inflammation(Payne *et al.*, 2017) (Hitchcock *et al.*, 2015). CLEC2 is a type II transmembrane protein expressed at a high level on platelets as non-covalent homodimers, with a hemITAM (a single YxxL motif) in its cytoplasmic tail.

1.3.5.2 Signalling of CLEC2

The transmembrane glycoprotein podoplanin is the only known physiological ligand of CLEC2. The signalling pathway bears a resemblance to GPVI signalling. Crosslinking of CLEC2 via ligation with podoplanin results in the activation of Src-family kinases leading the phosphorylation of the CLEC2 hemITAM. Phosphorylation of the CLEC2 hemITAM is different than for the phosphorylation seen in the ITAM of GPVI. For GPVI there are two phosphorylated tyrosine's within the ITAM to which Syk binds. For CLEC2, there is only one phosphorylated tyrosine in the hemITAM, as such Syk binds to binds two different CLEC2 cytosolic receptor chains. This leads to activation of Src, Syk, LAT, SLP-76, Gads and Btk (Manne *et al.*, 2015), which in turn activates PLCγ2. Positive feedback is essential in CLEC2 signalling through Rac, ADP and TXA_2 (Pollitt *et al.*, 2010).

The full role of CLEC2 in haemostasis in unknown, due to the only known physiological ligand being podoplanin, which is found in such tissues as metastatic tumour cells, the nervous system, and lymphatic endothelial cells (Ugorski, Dziegiel and Suchanski, 2016) (Rayes, Watson and Nieswandt, 2019). However, it has not been observed that podoplanin is present at the vascular wall, leading to questions as to how CLEC2 would activate in times of vascular injury. Nevertheless, mice which have blocked CLEC2 via a monoclonal antibody see a prolonged tail bleed time (May *et al.*, 2009), while another similar study argued there was no effect (Bender *et al.*, 2013). Platelets have been proposed to regulate the separation of lymphatic vessels and blood vessels via CLEC2 (Osada *et al.*, 2012).

As mentioned, CLEC2 plays a role in thrombo-inflammation. Studies have shown that hepatic sterile inflammation involves CLEC2 in liver acute toxic injury, due to macrophages expressing podoplanin (Chauhan *et al.*, 2020). Other studies have demonstrated in sepsis models that CLEC2 regulates the inflammatory response and stimulates the recruitment of macrophages. On top of this, in deep vein thrombosis and infection via *Salmonella*, CLEC2 initiates thrombus formation (Hitchcock *et al.*, 2015) (Payne *et al.*, 2017).

1.3.6 FcγRIIa

1.3.6.1 Structure of FcγRIIa

The FcγRIIa receptor, involved in immunity and found only in higher primates, is expressed on many cell types, such as monocytes and macrophages. Platelets express over 5000 copies of FcγRIIa per cell. Belonging to the class II FcγRs, this receptor is a single-chain type 1 transmembrane glycoprotein of around 40kDa. It has two extracellular Ig-like domains, the second of which mediates the binding to IgG, followed by a single transmembrane domain. The cytoplasmic domain contains an atypical ITAM, which, instead of the two sequences of (YxxL/I) being separated by six to eight amino acids, is separated by twelve amino acids (Brooks *et al.*, 1989). There is a common single amino acid polymorphism which gives rise to the two allelic forms of the FcγRIIa receptor. This G to A change results in the change of an arginine to a histidine at position 131. This has an impact on receptor binding efficiency, particularly towards the IgG subset IgG2 (Bournazos *et al.*, 2009). The receptor signalling is regulated by a high affinity for complexed IgG, and a low affinity for monomeric IgG. The FcγRIIa ectodomain region allows for dimerisation of the receptor upon ligand binding (Qiao *et al.*, 2015).

1.3.6.2 Agonists of FcγRIIa

The most common agonists of FcγRIIa activation include non-physiological agonists heat treated aggregated IgGs and beads coated in IgGs, and physiological agonists IgG opsonised pathogens, and heparin-induced thrombocytopenia (HIT) serum. The activation of FcγRIIa can result in the internalisation of IgG, and the activation of platelets in the form of aggregation, alpha and dense granule release, and the formation of microvesicles (Arman and Krauel, 2015). The murine IgG_{2b} monoclonal antibody (mAb), IV.3, which is specific for FcγRIIa can be used in FcγRIIa signalling. Used in its monomeric form, IV.3 mAb is inhibitory, binding the receptor in the second IgG-like domain, preventing other ligands from binding. IV.3 mAb can be crosslinked with secondary antibody $F(ab')_2$ fragments, resulting in the clustering of the receptor leading to platelet activation (Rosenfeld *et al.*, 1985).

Pathogens, such as viruses (e.g. influenza H1N1 (Boilard *et al.*, 2014)) and bacteria (e.g. *Staphylococcus aureus* (Fitzgerald *et al.*, 2006) *Streptococcus gordonii* (Arman *et al.*, 2014), and *Helicobacter pylori* (Byrne *et al.*, 2003)), can be opsonised by IgGs which

activates FcγRIIa. When antigens on the surface of the pathogen bind to the F(ab) region of IgG, the Fc region is recognised by FcγRIIa. Yet another way the FcγRIIa receptor can be activated is via immune complexes (IC). An example of which is in HIT, where the anticoagulant heparin and PF4 form a complex against which IgG antibodies are directed, which activate FcγRIIa (Horne and Alkins, 1996).

1.3.6.3 FcγRIIa signalling

Binding of multiple bacteria through the Fc region of IgGs to FcγRIIa induces receptor crosslinking, causing tyrosine-phosphorylation of the FcγRIIa ITAM by a Src-family kinase, such as Lyn or Fyn (Figure 1.5). This allows the docking of the SH2 domain-containing kinase Syk which results in Syk activation. Such activation is followed by the activation of LAT, which leads to the phosphorylation and activation of PLCγ2. Activated PLCγ2 increases the levels of PIP_2, the hydrolysis of which produces the increase in inositol-1,4,5-trispohosphate (IP_3) and diacylglycerol (DAG) resulting in an increase in intracellular calcium concentrations and the activation of PKC respectively, both of which are required responses for platelet activation, secretion, and aggregation. ADP is an essential cofactor of the FcγRIIa signalling pathway when secreted by platelets (Gratacap *et al.*, 2000). This secretion is important for a synergistic mechanism which also involves the Gi-dependant pathway of the P2Y12 ADP receptor (Figure 1.3) allowing for the efficient production of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) when FcγRIIa cross-linking occurs. Such signalling occurs in cell surface associated lipid rafts, where phosphorylated FcγRIIa is concentrated. Within lipid rafts P2Y12 production of PIP_3 occurs, catalysed by PI3-K. The production of PIP_3 is required in order to recruit PLCγ2 to lipid rafts, as well as other proteins such as Btk and protein kinase B (PKB), which allows for their phosphorylation and activation (Bodin *et al.*, 2003).

Important differences exist between activating FcγRIIa through antibody crosslinking or via bacteria. In response to bacteria, platelet aggregation takes place after a lag phase, and FcγRIIa phosphorylation and platelet secretion are dependent on αIIbβ3 engagement; moreover, this response is highly dependent on ADP and TXA_2 (Arman *et al.*, 2014).

Most cells express FcγRIIb, which has an immunoreceptor tyrosine-based inhibitory motif (ITIM), to counteract FcγRIIa (Daëron, 1997), but platelets do not. FcγRIIa downregulation is unknown in platelets, but it is thought that other ITIM receptors could be involved. One such receptor is platelet endothelial cell adhesion molecule-1 which is found to downregulate FcγRIIa-dependant processes and colocalises with FcγRIIa in the membrane (Thai *et al.*, 2003). It is known that *in vitro* cleavage of the ITAM sequence of FcγRIIa cytoplasmic domain occurs via Ca^{2+}-sensitive cysteine protease calpain (Gardiner *et al.*, 2008).

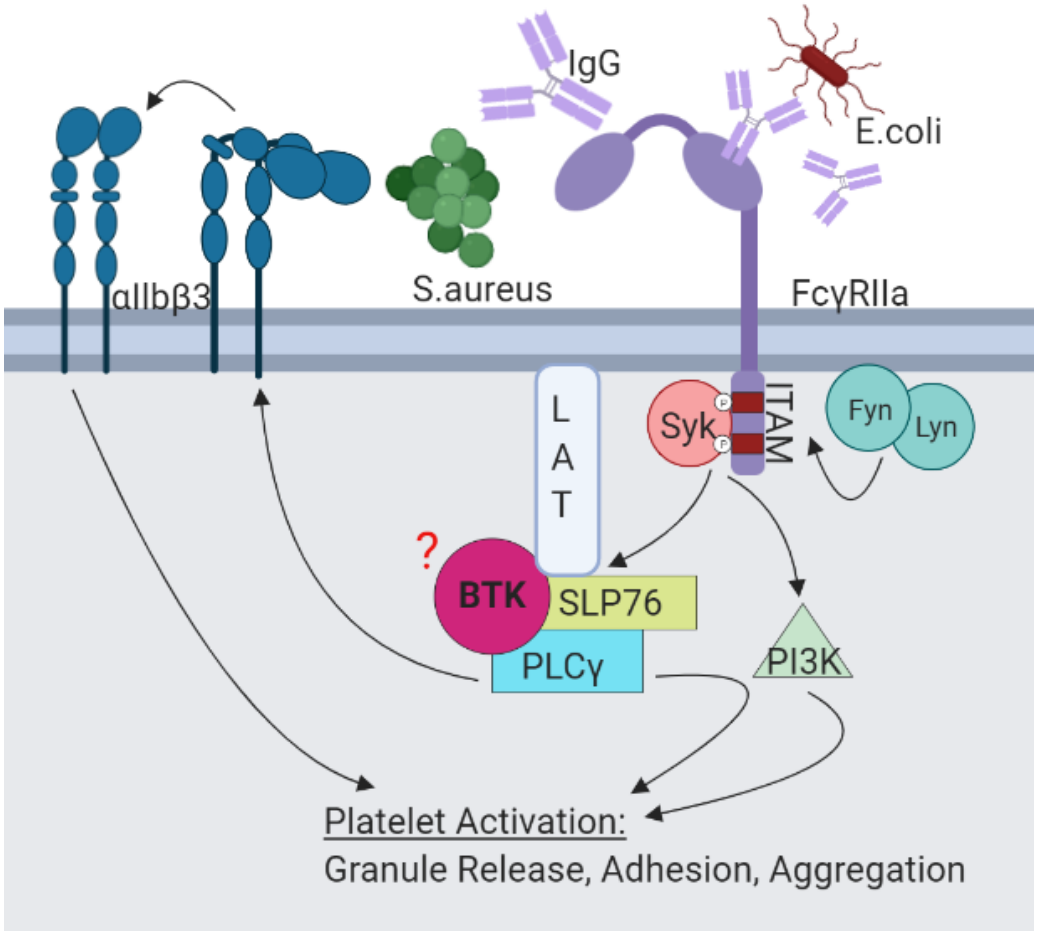


Figure 1.5. Platelet signalling through FcγRIIa in response to bacteria. Adapted from (Li et al., 2010). Question mark represents unknown of Btk activation downstream of FcγRIIa-mediated signalling in response to bacteria, as Btk has so far only been shown to be activated downstream of FcγRIIa when cross-linked with antibody.

1.3.6.4 Role of FcγRIIa in immunity

The FcγRIIa receptor is involved in autoimmunity, such as heparin-induced thrombocytopenia (HIT) that is caused by the formation of FcγRIIa-activating immune

complexes composed of IgGs, negatively charged heparin, and positively charged PF4, causing platelet activation. This results in a thrombotic disorder with thrombocytopenia (Arepally, 2017). Moreover, platelets are capable of internalising IgG immune complexes that bind to FcγRIIa (Worth *et al.*, 2006) which suggests that platelets can remove IgG complexes from the circulation.

FcγRIIa, in combination with PF4, has anti-microbial effects. PF4, released from platelet α-granules, is a positively charged tetramer, and as such can bind to anionic molecules on the surface of bacteria. When PF4 binds to bacteria, the protein undergoes a conformational change that exposes neoepitopes which are then recognised by anti-PF4 antibodies (Wolff *et al.*, 2020). The binding of the PF4-IgG complex to FcγRIIa results in the release of α-granules from platelets (Tardy *et al.*, 2020) which contain bactericidal molecules. Through these mechanisms, platelets have been shown to display a cytotoxic effect towards *Escherichia Coli* (*E. coli)* (Palankar *et al.*, 2018) and *Staphylococcus aureus* (*S. aureus)* (Ali *et al.*, 2017). However, other studies have found that while releasate from platelets was able to kill *S. aureus*, anti-PF4 antibodies only play a minor role (Wolff *et al.*, 2020).

In platelets, the FcγRIIa receptor has gained additional functions not related to recognising extracellular-IgG-related agonists. FcγRIIa is also involved in vascular integrity, contributing to αIIbβ3 outside-in signalling, to support formation of the thrombus (Boylan *et al.*, 2008). Moreover, FcγRIIa signalling improves collagen adhesion under flow, spreading, and aggregation *in vitro* (Boylan *et al.*, 2008) (Zhi *et al.*, 2013).

1.4 Platelet and Bacteria Interactions

1.4.1 Immunothrombosis

The best described role of platelets is their function in haemostasis. Vessel injury causes subendothelial collagen to become exposed, which platelets then interact with, as well as with collagen associated VWF. This leads to platelet activation and the recruitment of more platelets though the secretion of substances such as serotonin, TXA_2, ADP, and thrombin which results in platelet aggregation and clot formation. If

vessel injury is more extensive, TF expressed on adventitial cells and smooth muscle is exposed, which can then generate thrombin, resulting in platelet activation and thrombus formation (Varga-Szabo, Pleines and Nieswandt, 2008).

Immunothrombosis is the containment of pathogens in a thrombus formed of platelets, monocytes and neutrophils, or a fibrin fibre-rich thrombus, to protect endothelial integrity. Immunothrombosis is seen in a variety of conditions, including sepsis (Palankar and Greinacher, 2019), type 1 diabetes mellitus and graft versus host disease (Franchi *et al.*, 2019). One proposed mechanism of immunothrombosis is mediated by neutrophils with neutrophil extracellular traps (NETs). NETs contain histones and DNA matrix, are resistant to fibrinolysis, and are highly thrombotic. In this model, neutrophils activate in response to pathogens to form a NET which binds platelets (NETosis). The NETs contain elastase which promote coagulation by inactivation of the TF pathway inhibitor (Massberg *et al.*, 2010). This can be considered a variation of thrombosis induced by immune effector cells – immunothrombosis. The NETs have antimicrobial properties mediated by myeloperoxidase, matrix metalloproteinase 9 and peptidoglycan recognition protein 1 (Brinkmann *et al.*, 2004).

Immune cells such as monocytes promote the procoagulant function of platelets by releasing microparticles containing TF to which platelets respond by secreting protein disulphide isomerase which in turn activates TF to generate fibrin enhancing coagulation (Manukyan *et al.*, 2008). TF is also thought to be expressed by other cells such as neutrophils and platelets. Platelets also secrete other chemokines to activate immune cells, such as chemokine ligands 1, 4, 5, and 7.

Coagulation and thrombus formation can be deployed as a form of immediate, innate immunity. An example of this is mediated by fibrin, such as where *Streptococcus pyogenes* trapped in a fibrin clot underwent bacterial killing by platelets (Påhlman *et al.*, 2013), and mice lacking fibrinogen are susceptible to infection (Johnson *et al.*, 2003). Fibrin is also able to bind and activate multiple immune cells (Flick *et al.*, 2004) (Degen, Bugge and Goguen, 2007), as well as cytokines and chemokines (Szaba and Smiley, 2002). Therefore, immunothrombosis represents the formation of

microthombi in vessels to defend against pathogens, allowing the effective capture and killing of pathogens.

Mice which have severely reduced TF undergo a reduced coagulation response to bacteria, which results in an increased burden of infection and death (Luo *et al.*, 2011). TF gene expression is increased upon monocyte receptor recognition of pathogen associated molecular patterns, such as the common lipopolysaccharide. Intravascular TF is activated by the host cell exposing or releasing damage associated molecular patterns, such as phosphatidylserine and protein disulphide isomerase, which promotes coagulation. Platelets recognise bacteria, so this process, coupled with increased intravascular TF, jointly promotes coagulation. Platelets also interact with the complement system to promote coagulation, but it has also been reported that bacteria associate with platelets through GPIb as well as C3 complement protein (Verschoor *et al.*, 2011). This platelet bacteria association targets the immunologically important section of the bacterium to splenic $CD8\alpha^+$ dendritic cells which are involved in antigen cross-presentation and interact with macrophages to produce cytotoxic $CD8\alpha^+$ dendritic cell responses (Backer *et al.*, 2010).

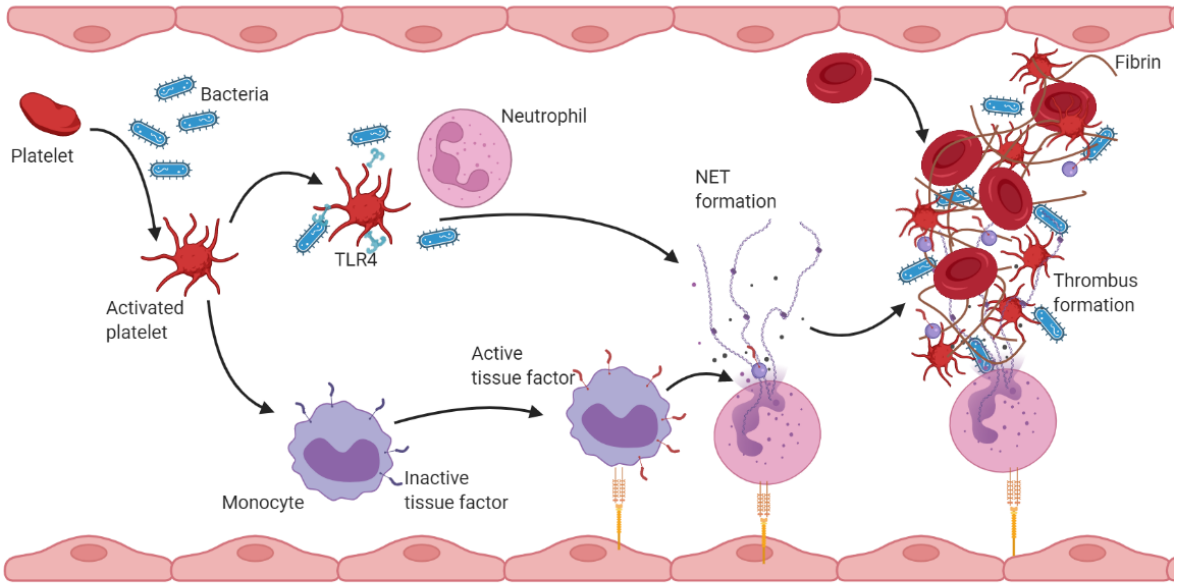


Figure 1.6. Events in occurring immunothrombosis. Adapted from (Duerschmied and Massberg, 2017).

1.4.2 Bacterial interactions with platelets

Platelets are known to interact with a wide range of bacteria, including *S. aureus* and *Streptococcus spp.* (Hamzeh-Cognasse *et al.*, 2015) These interactions can be classified in three broad mechanisms: the direct binding of the bacterium to platelet receptors; indirect binding of the bacterium to a plasma protein which will then bind to platelet receptors; and bacterially secreted products which can then go on to bind platelets (Cox, Kerrigan and Watson, 2011).

S. aureus causes a wide variety of diseases, usually skin infections, however it can also cause endocarditis and osteomyelitis. *S. aureus* expresses surface protein A (SpA) which, during growth, is secreted (Ton-That *et al.*, 1999). SpA consists of five immunoglobulin-binding domains which bind to IgG by its Fc domain, which has long been thought to prevent the opsonophagocytic killing of *S. aureus*. SpA can also bind to the Fab domain of IgG and IgM. On top of this SpA can cross-link with V_H3 type BCR which results in supralocal expansion and apoptosis of the B cell (Goodyear and Silverman, 2003). In addition, protein SpA can bind to VWF, which in turn binds to GPIbα resulting in platelet activation. This pathway can be partially inhibited using a VWF blocking antibody (Cox, Kerrigan and Watson, 2011).

S. aureus expresses the surface protein clumping factor A (ClfA) which binds fibrinogen. The C-terminal of fibrinogen allows binding to the peptidoglycan cell wall. Fibrinogen acts as a bridge between *S. aureus* and ClfA and platelet αIIbβ3 allowing for bacterium-platelet interaction to occur leading to platelet aggregation. However, if this receptor is blocked, platelet aggregation still occurs, suggesting an alternative pathway. Another surface protein of *S. aureus* is ClfB, which has a longer lag time to aggregation than ClfA and works through the same receptors. ClfA binds to the C-terminal region of the γ-chain of fibrinogen, while ClfB binds to the C-terminal region of the α-chain of fibrinogen. FcγRIIa and αIIbβ3 are involved in the interaction of ClfA and platelets (Kerrigan *et al.*, 2008).

There are instances of other bacteria also binding to fibrinogen, such as *S. pyogenes* by the M1 protein to activate αIIbβ3 (Shannon et al., 2007). In studied cases, the binding of bacterial proteins to αIIbβ3 (directly or via fibrinogen) does not cause stimulation

strong enough to result in platelet activation. Instead, further stimulus is needed in the form of FcγRIIa activation (Kerrigan, 2015).

Direct binding of αIIbβ3 can be achieved by some bacteria by the amino acid motif arginine glycine aspartate (RDG). One such example is *Staphylococcus epidermidis* which expresses the protein serine-aspartate repeat protein G. Serine-aspartate repeat protein G has been found to interact with platelets both indirectly through fibrinogen binding, and directly through αIIbβ3 (Brennan *et al.*, 2009). *S. aureus* express an RDG motif protein, IsdB, when concentrations of iron is limited which also directly binds αIIbβ3 (Miajlovic *et al.*, 2010). Platelet adhesion binding protein A is a protein expressed by *Streptococcus gordonii* which interacts directly with αIIbβ3 to mediate platelet adhesion (Petersen *et al.*, 2010).

Serine Rich Repeat (SRR) proteins, types of adhesin, are expressed by *Streptococcus gordonii* called Has and GspB, and *Streptococcus sanguinis* called SrpA (Kerrigan *et al.*, 2002) (Bensing, López and Sullam, 2004). All three proteins have a specific non-repetitive region which contains a Siglec domain which binds to sialic acids (Pyburn *et al.*, 2011) that is thought to be essential for interactions with GPIbα. *S. aureus* also expresses an SRR protein on its surface, SraP. However, this is not thought to bind to GPIb due little homology between non repeat regions compared to GspB, though the protein is involved with platelet binding (Siboo, Chambers and Sullam, 2005).

E. coli is another bacterium which interacts with platelets. Some specific strains of *E. coli* can induce apoptosis in platelets as evidenced by a reduction in mitochondrial potential, degradation of Bcl-X and the condensation of actin (Kraemer *et al.*, 2012a). This is thought to be mediated by the α-toxin of *E. coli* on calpain which is involved in Bcl-X degradation. *S. aureus* also has an α-toxin which is thought to have the same effect on platelets as that derived from *E. coli* (Kraemer *et al.*, 2012b). *E. coli* is also known to interact with platelets through FcγRIIa and αIIbβ3 (Watson *et al.*, 2016) which can also be enhanced by TXA_2 production and the complement (Moriarty *et al.*, 2016).

TLRs are immune receptors which bind to PAMPs (Armant and Fenton, 2002). Platelets mainly express TLRs 2, 4 and 9 (Cognasse *et al.*, 2005). TLR4 is implicated in platelet activation induced by *E. coli* (Matus *et al.*, 2017), a pathway which does not involve FcγRIIa. This route to platelet activation, though, maybe dependent on the strain of *E. coli*, as the previous experiments were performed with EHEC O111 only. Other *E. coli* strains, such as CFT073 and RS218, have been show to activate platelets through FcγRIIa, without the involvement of TLR4 (Watson *et al.*, 2016).

E. coli LPS has been found to activate platelets through TLR4 (Ståhl *et al.*, 2006) (Ståhl *et al.*, 2009) and potentiate platelet aggregation (Zhang *et al.*, 2009). Other studies have found LPS stimulation of platelet TLR4 results in an increase of NET formation (Clark *et al.*, 2007). However, other groups have found no platelet activation through TLR4 as a result of LPS (Ward *et al.*, 2005), as well as LPS causing the inhibition of aggregation in another study (Hashimoto *et al.*, 2009).

TLR2 binds to lipoteichoic acid. Lipoteichoic acid from *S. aureus* inhibits platelet aggregation (Sheu *et al.*, 2000), while *Streptococcus pneumoniae* allows platelet aggregation to occur through TLR2 utilising PI3K (Keane *et al.*, 2010). Lipoteichoic acid from *Streptococci* has been found to inhibit platelet aggregation in response to collagen (Beachey *et al.*, 1977). *S. epidermidis* activation of TLR2 by lipoteichoic acid allows platelet adhesion (Chugh *et al.*, 1990). Taken together, bacteria activation of platelet TLR2 can have a variety of effects.

Though bacteria have different mechanisms for binding, activation and aggregation of platelets, many Gram-positive and Gram-negative strains seem to share a common step that is the engagement of FcγRIIa. Blocking of this receptor inhibits platelet aggregation and secretion in response to multiple bacteria, including specific strains of *S. sanguinis, S. aureus, E. coli* and *Helicobacter pylori* (Arman *et al.*, 2014) (Moriarty *et al.*, 2016) (Byrne *et al.*, 2003). FcγRIIa is thought to colocalise with αIIbβ3 (Shido *et al.*, 1995) and GPIbα (Sullam *et al.*, 1998), which are required for the aggregation response.

1.4.3 Platelets and bacterial killing

Platelets are thought to induce bacterial killing in response to FcγRIIa ligation via the release of bactericidal substances. One study found that thrombin-induced platelet microbial protein (tPMP) from rabbit platelets binds to the cytoplasmic membrane of *S. aureus* in order to exert its microbicidal effects via cell lysis (Koo *et al.*, 1997). Thrombocidins, unlike tPMP, are not able to lyse the bacterial cell wall, however, they have been shown to kill a wide range of bacteria, such as: *S. aureus, E. coli, Lactococcus lactis,* and *Bacillus subtilis* (Krijgsveld *et al.*, 2000). In a rabbit model of infective endocarditis, with *Streptococcus sanguis* as the infective bacteria, releasate from thrombin activated platelets reduced bacterial numbers (Dankert *et al.*, 1995). Platelets also kill *Plasmodium falciparum* parasites within the infected red blood cells (McMorran *et al.*, 2009). It has also been found that upon platelet binding to red blood cell, PF4 is released. PF4 can bind to the Duffy antigen chemokine receptor on the red blood cell, which results in PF4 entering the cell. Here, PF4 can lyse the parasite (McMorran *et al.*, 2014).

In addition to the secretion of bactericidal substances, platelets have been shown to phagocytose pathogens. Some of the initial studies demonstrated the phagocytosis of latex beads by platelets. These studies also demonstrated the phagocytosis of platelets which had phagocytosed latex beads by leucocytes (Movat *et al.*, 1965). Platelets have been shown to engulf and possibly phagocytose bacteria such as *S. aureus* as well as viruses such as HIV (Youssefian *et al.*, 2002). In one theory, platelets spread their membranes over bacteria then contract, capturing the bacteria close to the granulomere of the platelet, allowing for the preferential release of α-granules towards the bacteria, generating a high concentration of anti-microbial substances (Palankar *et al.*, 2018). This theory is based on studies which discuss that is it unlikely that bacteria are phagocytosed and then transported to the α-granule inside the platelet in order for bacterial killing to take place (White, 2006), as well as other studies that demonstrate that platelets can orient granule release dependant on the spatial orientation of the matrix (Sakurai *et al.*, 2015).

Platelets have also been shown to inhibit the growth of *S. aureus* through mechanisms unrelated to FcγRIIa (Kraemer *et al.*, 2011) (Trier *et al.*, 2008). Another study found that at a high platelet concentration, platelets could inhibit the growth of *E. coli*

(Palankar *et al.*, 2018). However, this high platelet concentration was significantly reduced from over 500 platelets per bacterium to around 25 platelets per bacterium with the addition of PF4 and PF4/P antibodies. Inhibition of bacterial growth did not occur when FcγRIIa was blocked.

Migration and scavenging of fibrin(ogen) and fibrin-bound bacteria have recently been found to be a way in which platelets support immunity (Gaertner *et al.*, 2017). Recruitment of platelets to the site of infection allows the platelets to scavenge bacteria on top of triggering the activation of immune cells. The process in which platelets scan then scavenge specific components of their microenvironment requires actomyosin forces with the result of attachment of pathogens to the platelet surface. This attachment of pathogens could serve to bundle bacteria and aid other activated immune cells in bacterial killing.

1.5 Bruton's Tyrosine Kinase (Btk) and Pharmaceutical Inhibitors Ibrutinib and Acalabrutinib

1.5.1 Btk

Btk is a member of the second largest family of cytoplasmic non-receptor tyrosine kinases in mammals: the Tec family. Most of these kinases are found on haematopoietic cells (Smith *et al.*, 2001), with T cells expressing Tec, interleukin-2-inducible T-cell kinase (Itk) and Txk, while B cells express Tec, Itk and Btk, with the levels of Itk and Btk being higher than Tec in both B and T cells (Tomlinson *et al.*, 2004). Btk is an essential protein in B cells, involved in their development (mature B cells have lower levels of Btk (Nisitani *et al.*, 2000)), signalling and differentiation (Vetrie *et al.*, 1993). When a B cell is deficient in Btk, maturity is prevented. Deficiencies in Btk, either by lack of expression of loss of function, are found in some individuals and cause XLA. Patients with XLA have extremely low levels of B cells, as well as a reduction in all classes of immunoglobulins, so have an impaired immune response (Vetrie *et al.*, 1993). Mutations of Btk are mainly reported to affect B cells, though there are reports of other cells being affected too (Quek, Bolen and Watson, 1998) (Jongstra-Bilen *et al.*, 2008).

1.5.2 Btk structure

Btk is encoded on the X chromosome by 19 exons spanning 37.5kb (Sideras *et al.*, 1994). Btk has several domains which from the N terminal in sequence are: pleckstrin homology (PH) domain; Tec homology (TH) domain consisting of the Btk homology (BH) region and the polyproline region (PPR); Src-homology 3 (SH3) domain; Src-homology 2 (SH2) domain; and tyrosine kinase (Tk) domain. Each of these domains have been shown to interact with a variety of other proteins during signalling (Figure 1.7). Btk contains multiple regulatory phosphorylation sites: pS21, pS115, pS180, pY223, and pY551. Activity and stability of metalloprotein Btk requires Zn^{2+}, which it binds to through the zinc finger motif found in the TH domain (Vihinen, Nilsson and Smith, 1994).

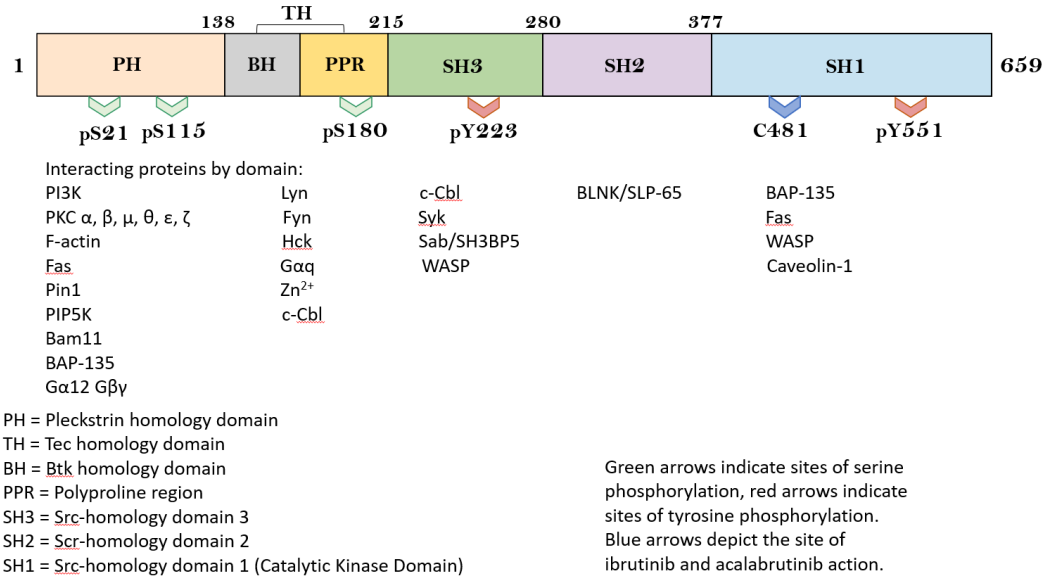


Figure 1.7. Schematic diagram of Btk highlighting domains, interacting proteins and sites of phosphorylation. Adapted from (Mohamed *et al.*, 2009).

1.5.3 Role of Btk in BCR signalling

When Btk is not activated it is in the cytosol. Upon BCR activation, Btk translocates to the plasma membrane where a Src-family kinase phosphorylates it at residue Y551. Y551 is in the catalytic domain and its phosphorylation causes the autophosphorylation of Y223 in the SH3 domain. Then, with the aid of the SH2 domain containing adaptor protein B cell linker protein, Btk phosphorylates PLCγ2 to begin its signalling pathway with the production of DAG and IP_3 (Hendriks, Yuvaraj and Kil,

2014). In CLL this pathway is amplified, and through effects on PI3-K, NFκB and PLCγ2, leads to the generation of pro-survival signals (Wen *et al.*, 2021). Due to its signalling to PI3-K, PLCγ2 plays a role in the maintenance of the tumour microenvironment. It has also been shown that Btk has a role in B cell homeostasis. This was demonstrated by the deletion of double stranded DNA ribonuclease Dicer in mice, which led to a decrease in the levels on miR-185 which plays a role in Btk post translational regulation, resulting in increased Btk, and an autoimmune phenotype (Belver, de Yébenes and Ramiro, 2010).

1.5.4 Role of Btk in innate immunity

More recently Btk's role in innate immunity has been explored. Though infection models with X-chromosome-linked immune-deficient (*Xid*) mice and with *Btk* gene knockout, it has been seen that Btk has a role downstream of receptors which sense a range of microbes like *S. aureus* in primary immune cells such as monocytes and macrophages (Liu *et al.*, 2017)*, Aspergillus faunigatas* by macrophages (Herbst *et al.*, 2015)*,* and *Listeria monocytogenes* by macrophages (Köprülü *et al.*, 2013).
Btk mediates signals generated by bacteria-immune cell interactions through TLR, specifically: TLR2 (Horwood *et al.*, 2006), TLR3 (Lee *et al.*, 2012), TLR4 (Horwood *et al.*, 2006), TLR7/8 (Li *et al.*, 2014) (Sochorová *et al.*, 2007) and TLR9 (Li *et al.*, 2014). A study of XLA patients confirmed the requirement of Btk in TLR signalling, with observations of impairments in TLR signalling being reported in dendritic cells (Taneichi *et al.*, 2008) (Sochorová *et al.*, 2007). Conversely, some studies claim contradictory results in neutrophils (Marron *et al.*, 2010), especially in the case of XLA patients, where, without functional Btk, TLR signalling would be expected to be impaired. This impairment in TLR signalling is thought to be due to specific Btk mutations arising in XLA. While the broad range of Btk mutations may allow for some functionality of TLR signalling, only certain Btk mutations may impact upon TLR signalling. Contradictory studies are also found about the role of Btk in phagocytosis, with studies in XLA patients showing both a redundancy in monocytes and neutrophils (Cavaliere *et al.*, 2017) (Marron *et al.*, 2010) and a requirement for Btk in monocytes and macrophages (Mirsafian *et al.*, 2017) (Braga Amoras *et al.*, 2003) (Di Paolo *et al.*, 2011).

Btk activation via TLR stimulates interferon-regulatory factor and NF-κB dependant transcription of interferons and cytokines involved in the inflammatory response (Jefferies *et al.*, 2003). Btk also encourages cytokine production through triggering receptor expressed on myeloid cells-1 myeloid receptor (Ormsby *et al.*, 2011). In XLA, the absence of Btk causes an upregulation of apoptosis-related genes, an increase in oxidative phosphorylation, and downregulation of genes involved in immunity (Mirsafian *et al.*, 2017).

The NOD- LRR- and pyrin domain-containing protein 3(NLRP3) inflammasome is key in the regulation of infection, stroke, diabetes, and myocardial infarction, due to the activation of IL-1β. In mouse macrophages, Btk interacts with the adapter protein apoptosis-associated speck-like protein containing CARD and NLRP3, resulting in the activation of the caspase-1 protein in the multicomplex. In peripheral blood mononuclear cells of XLA patients, the inflammasome was also impaired in terms of activity (Liu *et al.*, 2017), due to lack of Btk to phosphorylate and activate NLRP3 which impacts on the release of IL-1β (Weber, 2021).

Btk has a vital role in B cell development and maturation. Btk also is involved in the development of other cells. A deficiency in Btk, as well as Tec kinases, links to a decrease in macrophages and monocytes (Melcher *et al.*, 2008) in mice. Instead of monocytes and macrophages, granulocytes were instead formed, which were immature, had reduced efficiency of recruiting neutrophils, and whose granule function was inefficient. XLA patients have arrested neutrophils in the myelocyte or promyelocyte development stage (Farrar, Rohrer and Conley, 1996) (Winkelstein *et al.*, 2006). Dendritic cells of animals which also lacked Btk also had impaired antigen presentation and maturation (Kawakami *et al.*, 2006), which is also seen in human cells (Maurya *et al.*, 2014).

1.5.6 Btk inhibitors and their role in CLL

iBtks were first conceptualised for treatment of autoimmune diseases, however, when the original company who designed ibrutinib, Celera, sold assets to Pharmacyclics, ibrutinib was first explored in B cell cancers. Ibrutinib is the first generation of iBtks. Ibrutinib was first approved by the National Institute for Health and Care Excellence in

January 2017 for the treatment of CLL. Due to the pivotal role of Btk in BCR signalling, targeting Btk as a therapy for CLL was an obvious candidate. Ibrutinib is an irreversible Btk inhibitor with an IC_{50} *in vitro* of 0.5nM (Honigberg *et al.*, 2010). Ibrutinib achieves Btk inhibition by binding to the ATP-binding region of the TK domain of Btk at a cysteine in position at 481 (Honigberg *et al.*, 2010). Ibrutinib is also known to have an IC_{50} of 10, 96, 171 and 200nM for Tec, Fyn, Src and Lyn respectively (Honigberg *et al.*, 2010) (Barf *et al.*, 2017). For treatment in CLL, the drug is typically prescribed at a dose of 420mg once daily (Hardy-Abeloos, Pinotti and Gabrilove, 2020). Common side effects of the drug include diarrhoea, atrial fibrillation, increased infections, and bleeding (Stephens and Byrd, 2019).

In the RESONATE randomised trials, ibrutinib was compared to ofatumumab in patients who had been previously treated with at least one previous therapy for CLL or SLL. It was shown that ibrutinib improved progression free survival, overall survival and response rates (Byrd *et al.*, 2014). Ibrutinib is particularly effective in the high risk cases of p53 mutated CLL (O'Brien *et al.*, 2016). In a 2 year follow up of the RESONSATE study (Brown *et al.*, 2018), it was shown that patients having only one previous treatment had better outcomes than those who have over two previous treatments, similar to findings in other studies (Burger *et al.*, 2015). Ibrutinib has also been compared to chlorambucil in a randomised trial (Burger *et al.*, 2015), where ibrutinib was found to be more effective, with the 24 month survival rate at 98% rather than the 85% 24 month survival rate of chlorambucil, on top of this, the overall response rate was found to be 86% to chlorambucil's 35%.

Ibrutinib can be used in combination with other drugs. An example of this is venetoclax in the CLARITY trial for relapsed/refractory CLL (Hillmen *et al.*, 2017), and in another study of first line treatment (Jain *et al.*, 2017). This combination exploits ibrutinib's ability to increase the CLL cells sensitivity to venetoclax (Deng *et al.*, 2017), which is mediated through ibrutinib increasing the levels of Bcl2 like protein which interacts with other Bcl2 family members as well as promotes apoptosis. Though both trial durations have been short, they show promising results with many patients having negative minimal residual disease. However, longer follow ups would be needed.

Other Btk inhibitors are approved and in trials for B cell malignancies such as CLL as well as other diseases (Wen *et al.*, 2021). In CLL, two iBtks are of interest, acalabrutinib and zanubrutinib, which are both approved in Europe and the United States, and are amongst the most studied iBtks. Zanubrutinib is also approved in China. These second-generation inhibitors are being developed to be more specific for Btk to reduce adverse effects (Byrd *et al.*, 2016) (Wu, Zhang and Liu, 2016) (Tam *et al.*, 2020). Other iBtk include evobrutinib, fenebrutinib and tirabrutinib which are in trials. A comparison of these inhibitors is found in Table 1.2.

Acalabrutinib is a second-generation reversible inhibitor of Btk which has a similar structure to ibrutinib. It was approved for the treatment of CLL in March 2021. Acalabrutinib has a higher selectivity for Btk than ibrutinib, yet it has a higher IC_{50} at 5nM. This drug does not inhibit Src, Fyn and Lyn, unlike ibrutinib, and also has a higher IC_{50} for Tec at 126nM (Barf *et al.*, 2017). In CLL, acalabrutinib is prescribed at 100mg dose twice daily (Isaac and Mato, 2020). In phase II clinical trials, it was reported that acalabrutinib, though still causing gastrointestinal problems, caused no atrial fibrillation or bleeding (Byrd *et al.*, 2016).
Acalabrutinib was determined to have an overall response rate of 90% in both previously untreated CLL patients, and previously ibrutinib-treated CLL patients (Byrd *et al.*, 2016) (Byrd *et al.*, 2020) (Awan *et al.*, 2019) (Woyach *et al.*, 2020). The ELEVATE-TN study of 535 patients found acalabrutinib monotherapy is associated with better progression free survival compared to obinutuzumab-chlorambucil. Progression free survival of 93, 87 and 47% and treatment duration of 27.7 months, 27.7 months and 5.6 months for acalabrutinib-obinutuzumab-acalabrutinib, acalabrutinib monotherapy and obinutuzumab-chlorambucil respectively was found (Sharman *et al.*, 2020).

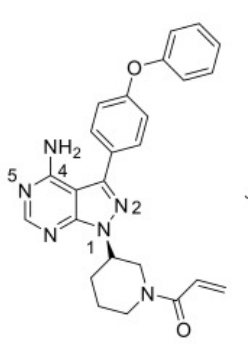
In the ASCEND trial of 310 patients, acalabrutinib was associated with improved progression free survival compared to idelalisib and rituximab treatment, which at 12 months was 88% versus 68% respectively, with overall response rates of 81 and 75% (Ghia *et al.*, 2020). The first head-to-head comparison of ibrutinib versus acalabrutinib was completed in 2021, with results showing acalabrutinib had non-inferior progression free survival, with less discontinuations and fewer toxicities such as atrial fibrillation (Byrd *et al.*, 2021).

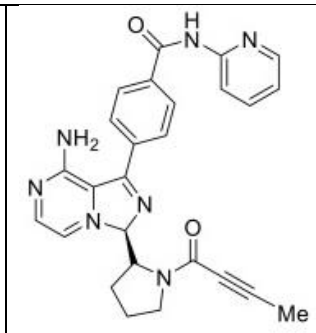
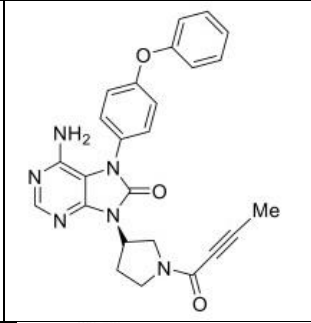
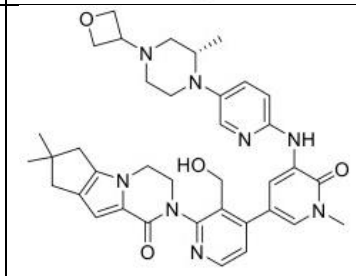
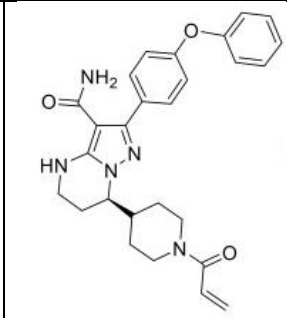
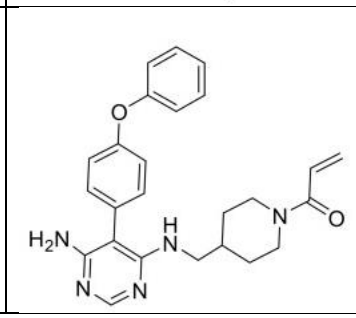
Zanubrutinib treatment is associated with an overall progression free survival rate of 91.2-100% (Tam *et al.*, 2019) (Xu *et al.*, 2019). Patients taking zanubrutinib tolerate the drug well, with normal Btk inhibitor associated toxicities being infrequent (Tam *et al.*, 2019). In a head-to-head trial comparing zanubrutinib to ibrutinib, zanubrutinib had better overall response rates, and toxicities were found to be less than ibrutinib (Hillmen *et al.*, 2019).

All the iBtks used in CLL management can have adverse side effects, such as leukocytosis. However, ibrutinib has also been reported to cause bleeding (Wang *et al.*, 2016) (Byrd *et al.*, 2013), including major bleeding events such as haematuria and subdural hematomas. This is due to the inhibition of Btk and Tec kinases, as well as other kinases, and the role they play in platelets. Ibrutinib and acalabrutinib treated patients have increased risk of infections too (Stephens and Byrd, 2019) (Byrd *et al.*, 2020). The development of resistance to ibrutinib and acalabrutinib has been reported which is thought to be due to an activation mutation in PLCγ2 or a Btk C481S mutation (Woyach *et al.*, 2014) (Woyach *et al.*, 2014) (Woyach *et al.*, 2017) (Hamasy *et al.*, 2017) (Quinquenel *et al.*, 2019) which would lead to an outgrowth of resistant CLL clones.

Due to off target effects of ibrutinib, further generations of iBtks were developed for use in CLL as well as other diseases such as autoimmune diseases and inflammation.

Table 1.2. Comparison of common iBtks

Btk Inhibitor	Structure	Potency	Reversibility	Off-Target Proteins
Ibrutinib		IC_{50} = 0.5nM	Covalent irreversible	Blk, Bmx, EGFR, HER2, HER4, Itk, Jak3, Txk, Tec

Acalabrutinib		IC_{50} = 3nM	Covalent irreversible	Bmx, HER4, Tec
Tirabrutinib		IC_{50} = 6.8nM	Covalent irreversible	Bmx, Txk, Tec
Fenebrutinib		K_i = 0.91nM	Non-covalent reversible	Bmx
Zanubrutinib		IC_{50} = 1.8nM	Covalent irreversible	Blk, Bmx, EGFR, HER4, Itk, Txk, Tec
Evobrutinib		IC_{50} = 8.9nM	Covalent irreversible	Bmx, Tec

Structure and potency from (Zhang, Gong and Meng, 2021); off-target proteins from (Estupiñán *et al.*, 2021).

1.5.6 Effect of ibrutinib and acalabrutinib on platelet haemostasis and thrombosis

Early clinical studies of ibrutinib noted an excess of bleeding events (Wang *et al.*, 2015) (Byrd *et al.*, 2015). In one study of 111 people treated with ibrutinib, 50% experienced

bleeding events and in 6% of these cases the bleeding was categorised as severe. In another study of 132 patients, bleeding events were observed in 61% of patients. In clinical studies, acalabrutinib did not cause major bleeding events, though low grade bleeding was still observed (Byrd *et al.*, 2016). In another study 26% of patients of 310 on acalabrutinib experienced a bleeding event (Ghia *et al.*, 2019).

In *ex vivo* studies of ibrutinib-treated patients the levels of the drug are enough to block Btk and reduce platelet aggregation to collagen related peptide (CRP). However, when acalabrutinib-treated CLL patient platelets underwent the same test, the reduction in aggregation in response to CRP was not seen (Nicolson *et al.*, 2018a). Other studies show that ibrutinib can have off-target effects on Src and Tec, whilst acalabrutinib is more specific for Btk and hence does not exert the same inhibitory effects on thrombus formation under arterial flow in response to collagen (Bye *et al.*, 2017). Ibrutinib-treated patients bleeding phenotype corresponds to a reduction in platelet aggregation in response to collagen (Kamel *et al.*, 2015) (Levade *et al.*, 2014) (Lipsky *et al.*, 2015) implicating the GPVI receptor pathway in ibrutinib inhibition.

Platelet adhesion to fibrinogen via αIIbβ3 is affected in ibrutinib-treated patients. Ibrutinib has been shown to inhibit outside-in signalling of αIIbβ3, which prevents calcium mobilisation, clot retraction and spreading. In the same study, mice deficient in αIIbβ3 outside-in signalling experience rebleeding in tail bleed experiments, and through this demonstrate that inhibition of αIIbβ3 outside-in signalling by ibrutinib could contribute towards the bleeding phenotype observed (Bye *et al.*, 2015a).

In vitro studies on blood from healthy donors demonstrated that pre-incubation with ibrutinib decreased, under sheer stress, the firm platelet adhesion to VWF (Levade *et al.*, 2014). It has been shown that patients taking ibrutinib who experienced bleeding have platelets which are less adherent to VWF, than platelets from patients on ibrutinib who lack a bleeding phenotype. VWF binds to the platelet receptor GPIb, which tethers platelets to vessels and is essential to thrombus formation, in a process where Btk is essential (Liu *et al.*, 2006).

Interestingly, ibrutinib has also been found to affect coagulation proteins in the blood. In a study which compared patients before ibrutinib treatment, and on day 28 of treatment, VWF and Factor VIII before treatment were found to be on the high end of the reference range but decreased upon ibrutinib treatment (Lipsky *et al.*, 2015). This decrease in VWF combined with a decreased ability of platelets to adhere to VWF may contribute to bleeding in ibrutinib treated patients.

Recent studies have found ibrutinib to influence the CLEC2 signalling pathway. Studies have shown that the CLEC2 pathway is heavily reliant on Btk, with low doses of ibrutinib and acalabrutinib completely abolishing signalling. This is opposite to what has been reported for GPVI in which GPVI activation can still occur due to compensation via Tec kinase (Nicolson *et al.*, 2021).

1.6 Infections in CLL and XLA

1.6.1 Infections in the general CLL population

CLL patients are predisposed to infective complications, with infections being the leading cause of death (Lee *et al.*, 1987) at 30-50% of deaths (Molica, 1994). In one study of 379 CLL patients (Molteni *et al.*, 2005), 7% of infections were fungal, 25% were viral, and 67% were bacterial. A key risk factor seen in this study was multiple lines of chemotherapy. However, there are disease related disease factors involved in this increased infection risk, in addition to immunosuppression of therapy.

An unavoidable factor involved in infection in CLL is that of disease stage. Patients at stage A are only 33% likely to contract an infection, while in stage C this number jumps to 82% (Itala *et al.*, 1992). At advanced disease stages, the lack of a functioning immune response increases the likelihood of mortality.

CLL patients have impairment in their neutrophils (Kontoyiannis *et al.*, 2013). As a result of chemotherapy and disease progression, neutropenia can occur, possibly allowing infections. In some CLL patients known to have repeated infections, it was found that there was low complement protein C3b compared with patients with no previous infections (Heath and Cheson, 1985). In addition, neutrophils in CLL patients prone to infection have defective migration, complement protein C5a stimulated

chemotaxis, and phagocytic function (Itälä, Vainio and Remes, 2009). Neutrophils are seen to be primed in CLL too. CLL patients release more NETs, with IL-8 in the plasma of CLL patients found to prime neutrophils for NET formation (Podaza *et al.*, 2017). Other studies have found neutrophils in CLL patients to be permanently primed, with increased production of reactive oxygen species and increased CD54 and CD64 which are markers of cell activation (Manukyan *et al.*, 2017).

T-cells are also impacted in CLL, in that colony forming capacity is affected, with a reduction in T-helper cells and an increase in T-suppressor cells. Programmed cell death ligands 1 and 2 expression is increased in CLL cells, resulting in an exhausted T-cell phenotype by supressing protein cell death ligand 1 expressing T cell immune responses (Ramsay *et al.*, 2012). Natural killer cells are also defective (Platsoucas *et al.*, 1982).

Another factor resulting in the increased risk of infections is hypogammaglobulinaemia. Hypogammaglobulinaemia, which can be related to the stage and duration of disease, affects 10-100% of CLL patients (Itala *et al.*, 1992) and is thought to be due to inhibitory cytokine activity from malignant B cells with the possibility of IL-10 having a role (Izcue, Coombes and Powrie, 2006). Increased severity and frequency of infections in CLL patients is linked to hypogammaglobulinaemia, resulting mainly in bacterial infections, with low levels of IgG being associated with *Streptococcus* infections (Nosari, 2012). However ultimately, immunoglobulins levels in CLL are reliant, whether low or normal, on the non-malignant B cells still being able to initiate an immune response.

1.6.2 Infections in ibrutinib-treated CLL patients

There have been many studies exploring the link between ibrutinib treatment and an increased risk of infections. Some reports state that infection incidence and neutropenia decline throughout ibrutinib treatment (Coutre *et al.*, 2019).
In one systematic review and meta-analysis (Ball *et al.*, 2020), a significant increase in mild infection risk was associated with use of ibrutinib in B cell malignancies. However, ibrutinib use was not associated with increased infection of any severity in CLL patients

treated with ibrutinib, yet high grade infections were significantly increased in this set of patients.

Another study analysed results from 378 patients with lymphoid cancer, most commonly CLL, receiving ibrutinib. This study found that within a year, 11.4% developed a serious infection, of with 37.2% were fungal, 9.3% were viral, and 14% resulted in death (Varughese *et al.*, 2018). In a retrospective study of lymphoid malignancies treated with ibrutinib, 52% developed an infection, 44% were hospitalised, and 2.9% died, with the median time to infection after the start of treatment being 70 days (Chaul Barbosa, DeAngelis and Grommes, 2017). Upper respiratory tract infections are the most common form of infection (Burger *et al.*, 2015) (Byrd *et al.*, 2013) (Chanan-Khan *et al.*, 2016) (Byrd *et al.*, 2014). However, one study suggested that although upper respiratory tract infection was one of the most commonly reported infections in ibrutinib-treated CLL, the risk of these infections was no higher than CLL patients not treated with ibrutinib (Ball *et al.*, 2020).

Relapsed/refractory patients are more likely to suffer more frequent and severe infections than treatment-naïve patients, with 51% to 13% with severe infections, 25% to 6% pneumonia, 7% to 0% sepsis, and 4% to 0% bacteraemia (Byrd *et al.*, 2015). Morbidity of infections decreases over time in patients taking ibrutinib, this is thought to be due to greater time surpassed from time of last chemotherapy, as well as the potential for ibrutinib to partially reconstruct the humoral immune response by increasing the levels of immunoglobulins, on top of levels of normal B-cells (Sun *et al.*, 2015).

In one study, *S. aureus* was found to be the most common bacteria found in CLL patients receiving ibrutinib treatment (Varughese *et al.*, 2018). The high rates of invasive fungal infections seen in ibrutinib-treated patients has been proposed to be related to macrophage and neutrophil Btk inhibition (Fiorcari *et al.*, 2020) (Estupiñán *et al.*, 2021).

1.6.3 XLA and infections

In XLA, the development of B cells is defective, which results in a primary immunodeficiency disease. The result of this is a profound reduction in the formation of antibodies and their function (Suri, Rawat and Singh, 2016). XLA arises from mutations in Btk, of which there are more than 600. These mutations cause either a non-functional Btk protein, or a complete absence of Btk (Nicolson *et al.*, 2018a). XLA presents in childhood with recurrent infections and is treated by prophylactic infusions of immunoglobulins to reduce the infection risk (Kanegane *et al.*, 2002).

XLA was first described by Colonel Ogden Bruton as a disorder characterised by pneumonia, recurrent sepsis and otitis media in a young boy (Bruton, 1953) (Bruton, 1952). XLA is characterised by a high infection risk particularly with encapsulated bacteria, with one study showing a probability of XLA patients developing chronic lung disease at 80% (Plebani *et al.*, 2002) with the most common causes of death being sepsis and chronic lung disease. Respiratory tract infections are the most prevalent form of infection, despite the use of immunoglobulin replacement therapy (Suri *et al.*, 2017) (Boushaki *et al.*, 2015) (Aadam *et al.*, 2016) (Van Der Hilst, Smits and Van Der Meer, 2002) (Hansel, Haeney and Thompson, 1987) (Chen *et al.*, 2016) (Winkelstein *et al.*, 2006). One Polish study reported that in 44 XLA patients gastrointestinal disorders were as common as respiratory tract infections (Pac *et al.*, 2017).

1.7 Aims of the Thesis

The Btk inhibitors ibrutinib and acalabrutinib are effective treatments for CLL. However, the irreversible inhibition of Btk results in unwanted side-effects due to Btk being expressed in other cell types as well as B cells. Moreover, in addition to Btk, ibrutinib can inhibit other tyrosine kinases. In platelets, ibrutinib inhibits GPVI, GPIb and αIIbβ3 signalling, and it is associated with a bleeding phenotype in CLL patients which can be severe. Impairment of these platelet signalling pathways by ibrutinib is thought to due to inhibition of Btk and targeting of off-target proteins.

Ibrutinib treatment in CLL is associated with an increased infection risk. The FcγRIIa receptor in platelets is, like GPVI, an ITAM receptor, and shares similarities in the involved signalling pathway, which includes Btk. FcγRIIa is an immune receptor in

platelets, and the role of Btk downstream of FcγRIIa activation by bacteria has not been studied. I hypothesised that Btk inhibitors impair the bacteria induced FcγRIIa intracellular signalling pathway in platelets, leading to reduced platelet responses to bacteria. Such reduced platelet responses in ibrutinib-treated patients could contribute to the increased risk of infections seen in CLL patients treated with this drug.

The main aim of this thesis is to investigate the role platelets play as innate immune effectors in CLL and how such responses are influenced by iBtk therapy.
The specific aims of this thesis are:

- To determine the *in vitro* effects of ibrutinib and acalabrutinib on FcγRIIa responses in platelets derived from healthy donors.
- To characterise platelet responses to bacteria and FcγRIIa crosslinking in treatment naïve CLL patients.
- To determine the effect of therapeutic ibrutinib-treatment on platelet responses to bacteria and FcγRIIa crosslinking in CLL patients.
- To investigate the requirement for Btk activation in platelet FcγRIIa-mediated signalling in response to bacteria, and the effect of ibrutinib and acalabrutinib on this signalling.

Chapter 2: Materials and Methods

2.1 Materials

Table 2.1. Primary antibodies for western blotting

Antibody Target	Mono/Polyclonal, Host	Supplier/Catalogue Number	Dilution Factor
Btk	Monoclonal, D3H5 (Rabbit)	Cell Signalling Technology (#8547S)	1:1000
pY223 Btk	Monoclonal, EP420Y (Rabbit)	Abcam (ab68217)	1:1000
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Monoclonal, 6C5 (Mouse)	Santa Cruz (sc-32233)	1:1000
GAPDH	Monoclonal, 14C10 (Rabbit)	Cell Signalling (#2118)	1:1000
Anti-phosphotyrosine	Monoclonal, 4G10 (Mouse)	Millipore (#05-321X)	1:1000

Table 2.2. Secondary antibodies for western blotting

Antibody	Host	Supplier/Catalogue Number	Dilution Factor
IR Dye 680CW anti-rabbit IgG, fluorescently labelled	Goat	Li-Cor (827-08365)	1:10000
IR Dye 680RD anti-mouse IgG, fluorescently labelled.	Goat	Li-Cor (827-08364)	1:10000
IR Dye 800CW anti-rabbit IgG, fluorescently labelled.	Goat	Li-Cor (926-68171)	1:10000
IR Dye 800CW anti-mouse IgG, fluorescently labelled.	Goat	Li-Cor (926-32210)	1:10000

Table 2.3. Antibodies for flow cytometry

Antibody Target	Mono/Polyclonal, Clone Host	Supplier/Catalogue Number	Dilution Factor
GPVI	Monoclonal, HY101, Mouse	BD Pharmingen™	1:40
GPVI Isotype Control	Monoclonal, MOPC-21, Mouse	BD Pharmingen™, 554121	1:80
FcγRIIa	Monoclonal, IV.3, Mouse	StemCell Technologies, #60012FI.1	1:5
FcγRIIa Isotype Control	Monoclonal, OKT4, Mouse	BioLegend, 317407	9:250
CD41	Monoclonal, P2, Mouse	Beckman Coulter, IM0649U	1:5
CD41	Monoclonal, 5B12, Mouse	DAKO, R7058	33:500
CD41 Isotype Control	Monoclonal, DAK-GO1, Mouse	DAKO, X0928	1:20

Table 2.4. Inhibitors and final concentration used

Inhibitor	Target	Supplier/Catalogue Number	Manufacturer	Final Concentration
IV.3 mAb	FcγRIIa	Bio X Cell (BE0224)	Bio X Cell	20µg/ml
Dasatinib	Src	Cell Guidance Systems (SM45)	Cell Guidance Systems	4µM
PRT-060318	Syk	Med Chem Express (HY-12974)	Med Chem Express	10µM
Eptifibatide	αIIbβ3	Gift from Prof Steve Watson (University of Birmingham)		9µM
Prostacyclin	Prostacyclin Receptor	Enzo Life Sciences (BML-PG011-0010)	Cayman Chemical	4µM
Ibrutinib	Btk	Cambridge Bioscience (16274)	Cayman Chemical	0.01-5µM
Acalabrutinib	Btk	Cambridge Bioscience (19899)	Cayman Chemical	1-15µM
Apyrase	ATP/ADP hydrolysis	Sigma (A6535-1KU)		2Units(U)/ml

Table 2.5. Activators and final concentrations used

Activator	Supplier/Catalogue Number	Manufacturer	Final Concentration
Crosslinked Collagen Related Peptide (CRP)	CambCol laboratories (XL-CRP)	CambCol laboratories	3µg/ml
TRAP6 (Thrombin Receptor Activator Peptide 6) amide	Cambridge Bioscience (H-2936-0005)	Bachem AG	3µM
ADP (Adenosine Diphosphate)	Sigma (A2754)	Sigma-Aldrich	10µM
IV.3 mAb	Bio X Cell (BE0224)	Bio X Cell	4µg/ml
F(ab')$_2$ Rabbit anti-Mouse IgG (H+L)	Invitrogen (PA5-33287)	Life Technologies	30µg/ml

Table 2.6. Bacterial species used

Bacteria Species	Source	Isolated from
***Staphylococcus aureus* Newman**	Prof Steven Kerrigan, Royal College of Surgeons in Ireland, Ireland	Secondarily infected tubercular osteomyelitis (Duthie and Lorenz, 1952)
***Escherichia coli* RS218**	Prof Ian Henderson, University of Queensland, Australia	Neonatal Meningitis (Silver *et al.*, 1980)
***Escherichia coli* CFT073**	Prof Ian Henderson, University of Queensland, Australia	Acute pyelonephritis and bacteraemia (Mobley *et al.*, 1990)
***Streptococcus Sanguinis* 133-79**	Prof Steven Kerrigan, Royal College of Surgeons in Ireland, Ireland	Bacterial endocarditis
***Streptococcus gordonii* DL1**	Prof Steven Kerrigan, Royal College of Surgeons in Ireland, Ireland	(Plummer and Douglas, 2006)
Streptococcus oralis CR834	Prof Steven Kerrigan, Royal College of Surgeons in Ireland, Ireland	Gum abscess (Willcox and Knox, 1991)

Table 2.7. Fluorescent dyes used

Dye	Stains	Supplier/ Catalogue Number
Hoechst 33342	dsDNA	Abcam (228551)
Tetramethylrhodamine B isothiocyanate (TRITC) phalloidin	F-actin	Sigma-Aldrich (P1951-1MG)

2.1.1. Chemicals

Unless indicated otherwise, materials and reagents used in this thesis were obtained from Sigma-Aldrich.

2.2 Methods

2.2.1 Ethics

Participants informed consent and ethical approval (HYMS Ethics 1501 for healthy controls, and NHS National Research Ethics 08/H1304/35 for patient samples) were obtained for this study. Research was conducted in line with the Declaration of Helsinki and with Good Clinical Practice for clinical research.

2.2.2 CLL and XLA patients

The diagnosis of CLL was in line with World Health Organisation guidelines, in which B lymphocytes in peripheral blood are greater than $5x10^9$/L and sustained for over 3 months, and flow cytometric assessment of these lymphocytes reveals a characteristic immunophenotype (low surface levels of CD20, CD79b and immunoglobulin, with the coexpression of B-cell antigens CD19. CD20 and CD23, and T-cell antigen CD5) (Hallek *et al.*, 2008). Patient treatment with ibrutinib was done in line with iwCLL guidelines (Hallek *et al.*, 2018) in which one of the following criteria needs to be met: autoimmune complications, disease-related symptoms such as weight loss, extranodal involvement, splenomegaly, lymphadenopathy, lymphocytosis or marrow failure. The dose of ibrutinib was 420mg daily, or 140mg daily in the case of two patients.

XLA was diagnosed in line with the European Society for Immunodeficiencies, with IgG concentrations of less than 200mg/dL in the serum, and IgM and IgA concentrations less than 20mg/dL, with repeated infections starting early in childhood. XLA patient 1 had the Btk gene sequenced via genomics of rare immune disorders (Simeoni *et al.*, 2018) and was found to have a missense mutation C to T in codon 28 in the PH domain of Btk. XLA patient 2 was found to have a mutation G to T at 240 base pairs in exon 3 of Btk. Both specific mutations were confirmed to be previously reported in the literature to be associated with XLA (Simeoni *et al.*, 2018) (Vihinen *et al.*, 1998) (Hussain *et al.*, 2011).

2.2.3 Collection of whole blood from healthy controls

For this study, platelets were freshly isolated from whole blood collected from healthy donors at the University of Hull. Healthy donors confirmed they had not taken medication known to affect platelet activity in the last 10 days. Whole blood was collected from drug-free volunteers via a 21-gauge needle and added to sodium citrate to prepare platelet rich plasma (PRP). For washed platelets, whole blood from healthy volunteers was taken in acid-citrate-dextrose (ACD).

2.2.4 Collection of whole blood from CLL and XLA patients

Platelets were freshly isolated from whole blood collected from CLL patients and XLA patients at Queen's Centre for Oncology and Haematology, Castle Hill Hospital, Hull and transported to University of Hull within 3 hours via BloodFast, a medical logistics company. For preparation of PRP, whole blood from patients was drawn into 3.2% sodium citrate vacutainers (BD, 369714). For washed platelet preparations, whole blood was drawn into ACD vacutainers (BD, 364816).

2.2.5 Preparation of platelets from healthy controls, CLL and XLA patients

For healthy controls, CLL and XLA patients, whole blood was then centrifuged at 190xg for 15 minutes at room temperature (approximately 20°C). The resulting upper layer was the PRP and was transferred to a fresh tube. The remaining PRP layer and red cells were centrifuged at 17000xg for 5 minutes to obtain platelet-poor plasma (PPP).

For the preparation of washed platelets, the PRP, with the addition of citric acid to a final concentration of 6mM and apyrase at 2U/ml was further centrifuged at 800xg for 12 minutes. The resulting pellet was resuspended in platelet wash buffer and apyrase at 2U/ml then centrifuged at 800xg for 12 minutes. The pellet was then resuspended in modified Tyrode's buffer, with the addition of calcium chloride to a final concentration of 1mM.

Platelets were counted via Z1 Coulter particle counter, and in the case of washed platelets, the concentrations were adjusted to those specified by the addition of modified Tyrode's buffer.

Platelet preparation buffers and solution, all filtered through 0.22µm and stored at 4°C:

Sodium Citrate:

124mM sodium citrate

ACD, pH 6.4:

113.8mM D-glucose anhydrous

29.9mM trisodium citrate dihydrate

72.6mM NaCl

2.9mM citric acid monohydrate

Wash Buffer, pH 6.5:

36mM citric acid monohydrate

10mM EDTA (ethylenediaminetetraacetic acid)

5mM D-glucose anhydrous

5mM KCl

90mM NaCl

Modified Tyrode's Buffer, pH 7.4:

20mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

134mM NaCl

2mM KCl

0.34mM NaH_2PO_4

12mM $NaHCO_3$

1mM $MgCl_2$

5mM D-glucose anhydrous

2.2.6 Preparation of bacteria

Escherichia coli RS218 was grown aerobically in lysogeny broth (LB), and *Staphylococcus aureus* Newman and all other bacteria were grown anaerobically in Brain Heart Infusion (BHI) media, all at 37˚C overnight. Bacteria were washed by 4000xg centrifugation for 10 minutes and resuspended in pH7.4 PBS (137mM NaCl, 2.7mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4) to an optical density 1.6 at a wavelength of 600nm for light transmission aggregometry, enzyme linked immunosorbent assay (ELISA) supernatant collection, ATP release and lysate collection.

Bacterial suspensions at an optical density of 1.0 at a wavelength of 600nm were used for scavenging assays.

2.2.7 Colony forming units of bacteria

Colony forming units (CFU) were determined by serially diluting washed bacteria and plating on BHI agar plates for *S. aureus* Newman, and on LB agar plates for *E. coli* RS218. Plates were grown overnight at 37˚C. Colonies were then manually counted and the concentration of CFUs calculated.

2.2.8 Light transmission aggregometry

Light transmission aggregometry (LTA) is the 'gold standard' platelet function test for assessing *in vitro* platelet-platelet aggregation (Hvas and Favaloro, 2017) (Frontroth, 2013). Introduced in the 1960s, LTA is widely used in diagnostic work for patients. The technique uses the transmission of light through a sample to determine how optically dense it is. Upon platelet activation, a slight decrease in light transmission can be seen as platelets change shape, this is then followed by increased transmission of light through the sample as the platelets form aggregates (Figure 2.1).

LTA was performed in a Model 490 4+4 aggregometer (Chronolog, Havertown, PA) in 250µl final reactions. Washed platelets at a concentration of $2.5x10^8$/ml, or undiluted PRP was added to siliconised glass cuvettes with a stirring magnet and warmed to 37˚C in still conditions. Platelets were incubated with inhibitors for specific times (as indicated in the results sections of result chapters) in still conditions. Agonist was added under stirring conditions of 1000rpm, and aggregation was measured for either 10 or 20 minutes depending on the agonist used. If using washed platelets, reactions were supplemented with fibrinogen (1mg/ml fibrinogen that was plasminogen, von Willebrand Factor and fibronectin depleted) (Enzyme Research Laboratories) and pooled IgGs from human serum (0.2mg/ml) (I4506, Sigma-Aldrich, Missouri, USA). Blanks used for the aggregometer were either PPP (for PRP reactions) or modified Tyrode's buffer (for washed platelet reactions).

2.2.9 Dense granule release

Measurement of ATP released to the extracellular medium can be used as a read-out of platelet dense granule release, due to platelet ATP being stored in dense granules (McNicol and Israels, 1999). Luciferin and luciferase, both provided in the ChronoLume™ kit (D-luciferin-luciferase containing commercial reagent) (Chronolog, Havertown, PA) are added to the sample. ATP in the sample is used for the oxidisation of luciferin by luciferase. This reaction produces light, the quantity of which is proportional to the amount of luciferin oxidised and hence the amount of ATP in the sample.

Samples were prepared as indicated in section 2.2.5, and ATP was measured 5 minutes after the start of aggregation was seen (Figure 2.1) or equivalent time in the case of inhibitors. For this, 90µl of sample were plated in duplicate in 96-well plates, and 10µl of ChronoLume™ was added to each well in the dark. The plate was then read for luminescence in a Tecan infinite™ M200 plate reader with an integration time of 200ms. ATP release from addition of 12µM TRAP6 amide was considered 100% ATP release as this is maximal platelet activation and was measured for each donor.

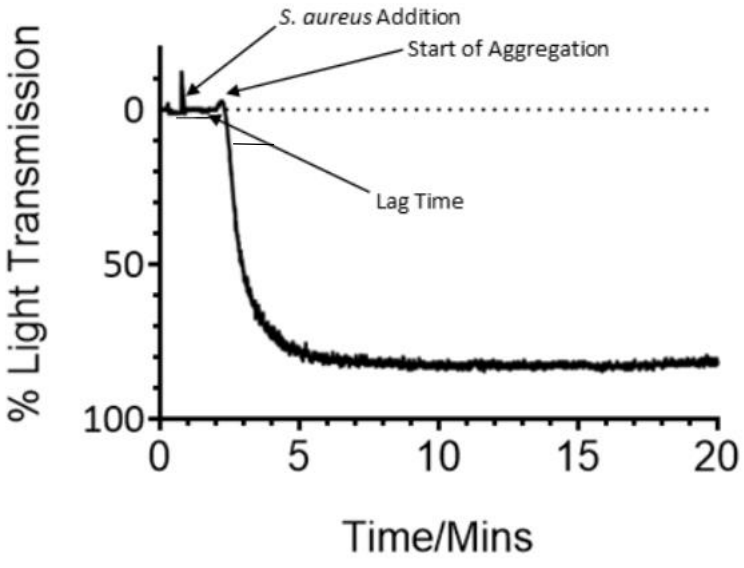


Figure 2.1. Graph depicting a typical light transmission aggregometry trace of PRP and S. aureus Newman. Reactions were monitored for 20 minutes. Arrows highlight the addition of agonist and perceived start of aggregation.

2.2.10 α-granule release

Measurement of PF4 released from α-granules to the extracellular medium by ELISA can be used as a read-out of platelet α-granule release. The ELISA kit used in this study was a human CXCL4/PF4 kit (DY795, R&D systems, Minneapolis, USA) but with minor modifications in the samples preparation to enable the detection of PF4 in the presence of plasma (Arman *et al.*, 2014). The kit works as a sandwich ELISA in which two anti-PF4 antibodies are used to sandwich the antigen. A capture antibody which binds PF4 coats the wells of a plate. The sample is added to the wells, and PF4 from the sample will bind to the capture antibody. After washing the unbound sample, the plate is blocked to prevent non-specific binding of the detection antibody to the wells. The anti-PF4 detection antibody conjugated to biotin is added to the wells, followed by streptavidin-horseradish peroxidase (HRP) that binds to biotin. A substrate solution (tetramethylbenzamide (TMB)) is added to detect HRP and a colour reaction occurs. A stop solution of sulphuric acid terminates the reaction by changing the pH of the system. The samples light absorbance is measured by a plate reader. A 4-parameter fit of known PF4 standard concentration absorbances allows for the calculation of sample concentrations of PF4.

In this study, samples were collected as follows: after the full-length aggregation reaction described in section 2.2.8, prostacyclin was added to each cuvette to a final concentration of 4µM to prevent further platelet activation. Samples were then removed from cuvettes, placed into Eppendorf tubes®, and centrifuged at 1500xg at room temperature. The supernatant was then removed and stored at -20˚C.

A 96 well plate was coated with PF4 capture antibody (2µg/ml mouse anti-human PF4 antibody reconstituted in 1% bovine serum albumin (BSA)-PBS (R&D systems Minneapolis, USA)), sealed with an adhesive strip, and incubated overnight at room temperature. Wells were then washed three times with washing solution (0.05% Tween20-PBS, pH7.4) before the addition of blocking solution (10%BSA-PBS). Plates were sealed with an adhesive strip and incubated at room temperature for 1 hour. Wells were washed three times with washing solution before sample addition. Plasma samples had been thawed and diluted to 1:10000 for clinical plasma and 1:20000 for healthy volunteers (as determined by optimisation to fit in scale), in reagent diluent

(50% foetal bovine serum-PBS). Plates were sealed with an adhesive strip and incubated for 2 hours at room temperature. Wells were washed three times with washing solution. Detection antibody (biotinylated polyclonal goat anti-human PF4, R&D systems, Minneapolis, USA, reconstituted in 1% BSA-PBS) was added to each well, before the plate was sealed with an adhesive strip and incubated at room temperature for 2 hours. Wells were washed three times with washing solution. Streptavidin-HRP (R&D systems, Minneapolis, USA) reconstituted in 1% BSA-PBS was added to each well. The plate was sealed with an adhesive strip and incubated at room temperature for 20 minutes in the dark. Wells were washed three times with washing solution before the substrate solution (solution A (H_2O_2) and solution B (TMB) in a 1:1 ratio, R&D systems, Minneapolis, USA) was added to each well. The plate was sealed with an adhesive strip and incubated at room temperature for 20 minutes in the dark. Stop solution ($2NH_2SO_4$, R&D systems, Minneapolis, USA) was added to each well.

The plate was read immediately at 450nm with a correction wavelength of 570nm by a BMG Labtech, FLUOstar® Omega plate reader (BMG Labtech, Ortenberg, Germany). Each sample was plated in duplicate, and PF4 standard (recombinant human PF4, R&D Systems, Minneapolis, USA, reconstituted in 1% BSA-PBS) was included in every assay in duplicates too. Sample concentrations were determined by a four-parameter logistic curve-fit of PF4 standards.

2.2.11 Protein phosphorylation

To investigate platelet intracellular signalling, protein phosphorylation was studied in whole cell lysates by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and LiCor near-infrared (NIR) western blot detection. SDS-PAGE is a commonly used technique to separate specific proteins of interest from a mixture. The ionic detergent SDS reduces a protein to its subunits by intercalating hydrophobic sites of the protein. B-mercaptoethanol further disrupts a proteins structure by breaking disulphide bonds. Upon the addition of β-mercaptoethanol and SDS to a protein, the protein becomes negatively charged due to the binding of SDS to the protein. An electric current can be applied to the mixture in a polyacrylamide gel, allowing the protein to separate from other proteins in a mixture by its molecular size, with the smallest of proteins migrating faster in the gel. Western

blotting allows the transfer of the proteins from a polyacrylamide gel onto a membrane. This membrane contains protein bands from the gel which can be detected on the membrane by antibodies. A primary antibody against the protein of interest is first used to probe the membrane before a secondary antibody raised against the source of the primary antibody is used. This secondary antibody is conjugated to a NIR dye. The fluorescence of this dye is then detected by Licor Odyssey CLx imaging system (LI-COR laboratories, Cambridge, UK).

Samples for protein phosphorylation studies were collected 3 minutes after platelet activation in PRP for crosslinked IV.3 mAb, CRP and TRAP6 amide, and at maximum aggregation for bacterial agonists. Aggregation reactions were stopped by adding a 1:1 ratio of cold PBS, the samples were transferred from cuvette to a 1.5ml Eppendorf tube®. Platelets were pelleted by centrifugation at 8500g at 4˚C for 50 seconds, then pellets were lysed via cold 1x lysis buffer (150mM sodium chloride, 10mM tris base, 1mM EGTA (ethylene glycol tetraacetic acid), 1mM EDTA, 2.5mM sodium orthovanadate, 0.1mg/ml AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), 5µg/ml leupeptin, 5µg/ml aprotinin, 0.5µg/ml pepstatin, 2% IPEGAL CA630, pH 7.5), with a lysis buffer volume equivalent to $5x10^8$ platelets/ml. Lysates were boiled at 95˚C for 5 minutes to denature samples then centrifuged at 4˚C at 14000xg for 10 minutes. Samples were run on a 10% polyacrylamide gel (Table 2.8) at 120V for 90 minutes before the gel was transferred onto a polyvinylidene fluoride (PVDF) membrane using the Bio-Rad Trans-blot turbo system with a kit-specific transfer buffer (Bio-Rad, Hertfordshire, UK) for 10 minutes at 2.5A 25V. The PVDF membrane was washed once in Tris-buffered saline (TBS) (20mM Tris-base, 0.137M sodium chloride, pH 7.6), before blocking for 1 hour in 5% BSA-TBS with 0.1% sodium azide.

Membranes were incubated with primary antibodies, D3H5 rabbit mAb for total Btk detection and EP420Y rabbit mAb for Btk pY223 detection, overnight at 4°C. After Btk/pBtk detection, the same membranes were incubated for 1 hour at room temperature with 6C5 mouse mAb for GAPDH as a loading control. Membranes were washed three times in TBS with 0.1% Tween (0.1% TBST), before the incubation with NIR conjugated antibodies (Li-Cor IRDye 800CW goat anti-rabbit IgG or Li-Cor IRDye

680CW goat anti-mouse IgG, both at 1:10000 in 0.1% TBST) for 1 hour. Membranes were then washed three times in 0.1% TBST before being placed in TBS. Membranes were imaged on the Odyssey-CLx imaging system (LI-COR laboratories, Cambridge, UK) at the wavelength corresponding to the secondary antibody used (800nm for the rabbit primaries anti-rabbit secondary combination, and 680nm for the mouse primaries anti-mouse secondaries combinations). Analysis of banding was performed in the Image Studio™ Lite version 5.2 software (LI-COR laboratories, Cambridge, UK).

Table 2.8. Preparation of 10% SDS Polyacrylamide Gel

10% Resolving Gel	**3.75% Stacking Gel**
3.33ml 30% acrylamide (37.5:1 acrylamide:crosslinker)	0.625ml acrylamide (37.5:1, 30%)
1.25ml resolving gel buffer (3M Tris-HCl, pH 8.8)	1.25ml stacking gel buffer (0.5M Tris-HCl, pH 6.8)
50µl 20% SDS	25µl 20% SDS
4.86ml distilled water (dH_2O)	2.85ml dH_2O
5µl tetramethylethylenediamine (TEMED)	4µl TEMED
500µl 1.5% ammonium persulphate (APS)	500µl 1.5% APS

2.2.12 Tec ELISA

To measure relative Tec phosphorylation levels, a sandwich-based ELISA was performed via Raybio® Human Phosphotyrosine (RayBiotech, Georgia, USA) TEC ELISA Kit. Samples were collected from washed platelets at $1x10^9$ platelets/ml supplemented with human IgGs (hIgGs) and fibrinogen as specified in section 2.2.8 and stimulated with either CRP, *S. aureus* Newman or *E. coli* RS218 in a light transmission aggregometer. Samples were collected at 3 minutes after start of aggregation for CRP reactions, and at maximum aggregation for bacterial agonists, or at a parallel time point in the case of inhibition. At these time points, samples were lysed in 2x lysis buffer (300mM sodium chloride, 20mM tris base, 2mM EGTA (ethylene glycol tetraacetic acid), 2mM EDTA, 5mM sodium orthovanadate, 0.2mg/ml AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride), 10µg/ml leupeptin, 10µg/ml aprotinin, 1µg/ml pepstatin, 2% IPEGAL CA630, pH 7.5) to a final concentration equivalent to $5x10^8$ platelets/ml.

Lysates were diluted five-fold in assay diluent, added to wells of a 96-well plate precoated with an anti-Tec antibody, and incubated for 2.5 hours at room temperature with gentle shaking. Wells were then washed and a biotinylated anti-phosphotyrosine antibody added for 1 hour at room temperature with gentle shaking. Wells were washed and incubated with HRP-conjugated streptavidin for 45 minutes at room temperature. Wells were washed and TMB substrate solution was added for 30 minutes at room temperature. Stop solution was then added to each well. Plates were read at 450nm BMG Labtech, FLUOstar® Omega plate reader. Each sample was plated in duplicate. Sample concentrations were determined by a four-parameter logistic curve-fit of positive control phospho-Tec from K562 cell lysates contained in the ELISA kit, which were included in each experiment and plated in duplicate.

2.2.13 Platelet scavenging and spreading

Platelets are known to be able to scavenge bacteria, and as such, scavenging experiments were carried out to investigate the effect of ibrutinib on this parameter. Fluorescence microscopy was used to measure platelet scavenging of surface-bound *S. aureus* Newman. Moreover, platelet spreading on fibrinogen assays were run in parallel as controls.

S. aureus Newman coverslip preparation:

Coverslips were placed in a humid chamber, coated in poly-L-lysine solution (Sigma-Aldrich, Missouri, USA) diluted 1:1 for a final concentration of 0.005% with dH_2O for 15 minutes before being washed twice with PBS. Bacteria, prepared in section 2.2.6 and adjusted to an optical density 1.0 at a wavelength of 600nm, were added to coverslips for 1 hour at room temperature. Unbound bacteria were aspirated from coverslips. Coverslips were washed once with PBS before the addition of 0.5% heat inactivated BSA for 1 hour. Unbound BSA was then aspirated, and coverslips were washed twice with PBS.

Fibrinogen coverslip preparation:

For fibrinogen spreading controls, fibrinogen at 100µg/ml was added to coverslips for 1 hour at room temperature. Fibrinogen was removed and coverslips were washed once with PBS before the addition of 0.5% heat inactivated BSA for 1 hour. Unbound BSA was then removed, and coverslips washed twice with PBS.

Fixing and Staining of Cells:

For *S. aureus* Newman coverslips, PBS was removed before the addition of PRP diluted with Tyrode's buffer and autologous plasma (prepared as in section 2.2.5) to a final concentration of $2x10^7$ platelets/ml with 15% plasma total followed by incubation for 1 hour at 37˚C.

For fibrinogen coverslips, PBS was removed before the addition of platelets either diluted in Tyrode's buffer and a final 15% plasma concentration (to control for bacteria scavenging assays) for 1 hour at 37˚C.

Unbound platelets were removed from coverslips and coverslips were washed with PBS. 4% paraformaldehyde was added to coverslips at room temperature for 10 minutes followed by two washed with PBS. 0.3% Triton X-100 was added to coverslips for 5 minutes. Coverslips were again washed twice with PBS. Fluorescent dyes Hoechst 33342 (stains DNA) and TRITC phalloidin (stains actin structures) were added to the coverslips and incubated for 20 minutes before the coverslips were washed twice with PBS.

Mounting of Coverslips

Coverslips were washed once in dH_2O. Coverslips were then picked up gently with tweezers and the excess dH_2O was removed by touching the edge of the coverslip to a paper towel. The coverslip was placed on top of a drop of ProLong Diamond Antifade Mountant (GE Healthcare, Illinois, USA) on a glass slide so that the coated side of the coverslip was facing the glass. Slides were labelled and stored in the dark to dry prior to fluorescence microscopy imaging.

Microscopy and Image Analysis

Images of the coverslips were taken by a Zeiss Axio Observer fluorescence microscope (Carl Zeiss, Cambridge, UK) with a x40 oil immersion objective and an AxioCam 506 camera. Images of coverslips were taken at random. ImageJ (NIH, USA) and Zen Pro software (Carl Zeiss, UK) were used to process and analyse all images. Z stacks were taken where the upper and lower limits are defined when the top and bottom of the platelet were out of focus, respectively. Z stack slices were taken 0.2µM apart, which

averaged 80 slices per z stack. All blood samples were blinded upon arrival in which another lab member would change the sample number to a number 1 through 4. Said lab member would note the sample number to the new corresponding number. The sample number was only revealed after analysis of spreading was completed.

For bacteria scavenging assays, calculating the number of bacteria in a cluster was achieved by first calculating the background intensity per pixel. This was done by circling 20 areas in which no bacteria were found and dividing the raw intensity by the area (the number of pixels). Results were averaged. 50 bacteria were then circled and the average intensity of each was calculated by subtracting the related background (number of pixels circles multiplied by background intensity per pixel). The results were averaged. Every cluster per image was then circled and the background was subtracted from the intensity of the cluster. This value was then divided by the average intensity of a single bacteria to calculate the number of bacteria per cluster.

2.2.14 Flow cytometry analysis

Flow cytometry was utilised in this study to ascertain cell surface receptor levels on platelets of healthy controls, CLL patients and ibrutinib-treated CLL patients. During flow cytometry, a flow of single cells passes through laser beams and the interaction with the light is measured in terms of fluorescence intensity and light scatter.

PRP was diluted to $1x10^7$ platelets/ml in modified Tyrode's buffer and was incubated for 20 minutes in the dark with antibodies, isotype controls or left unstained. For GPVI receptor assessment, PE-conjugated anti-GPVI (clone HY101; BD Pharmingen) and mouse IgG1κ control (clone MOPC-21; BD Pharmingen, California, USA) were used. For FcγRIIa assessment, anti-CD32 (FcγRIIA) (clone IV.3; StemCell Technologies, Vancouver, Canada) and anti-CD4 (clone OKT4, a mouse IgG2b against an irrelevant antigen for platelets; Biolegend, California, USA) were used. For αIIbβ3 (CD41/CD61) detection, two CD41 antibodies were used as technical replicates: RPE-conjugated anti-CD41 (clone 5B12; DAKO) and mouse IgG1 control (DAK-GO1; DAKO, Hovedstaden, Denmark); and FITC-conjugated anti-CD41 (clone P2; Beckman Coulter, California, USA).

Platelets were fixed by the final concentration of 0.45% cold PFA and kept on ice until analysis on the same day with BD LSR Fortessa cell analyser (BD Bioscience, New Jersey, USA). Samples were collected over 6 weeks, with a healthy control sample used each experimental day, using the same configuration baseline validated with daily CS&T procedures. Moreover, 8-peak Rainbow Calibration Particles (3.2µm average size) (BD Biosciences, New Jersey, USA) were used to standardise target reference values. In each experimental condition, 10000 events in the platelet gate were read from each sample by the FACSDiva™ Software (BD Biosciences, New Jersey, USA). Data acquired was then analysed in FlowJo software (BD Biosciences, New Jersey, USA).

2.2.15 Statistics

Statistical analysis was achieved by first determining the data distribution via the D'Agostino-Pearson omnibus K2 normality test. For data having a Gaussian distribution, one- or two-way ANOVA tests, with statistical significance set to $p<0.05$ were performed as required based on the number of independent variables being compared. If a Gaussian distribution was not found, then a Freidman test was carried out, with statistical significance set to $p<0.05$. For patient characteristics statistical analysis, Fisher's exact tests and chi-square tests were performed for categorical variables, while Mann Whitney and T-tests were performed for non-parametric and parametric continuous variables respectively, in all cases statistical significance was set to $p<0.05$. To ascertain statistical significance between experimental conditions and to correct for multiple comparisons, Tukey's post-hoc analysis was performed unless otherwise indicated. All statistics was performed in GraphPad Prism™ 8 software (GraphPad, California, USA).

Chapter 3: Ibrutinib and Acalabrutinib Inhibit FcγRIIa-Mediated Platelet Responses to Bacteria in Platelets Derived from Healthy Donors

3.1 Introduction

iBtks are used for the treatment of CLL, mantle cell lymphoma and Waldenstrom's macroglobulinemia (Burger, 2019). These drugs exert their effects by the downregulation of pro-survival BCR signalling in B cells through the inhibition of Btk. Ibrutinib is a first generation iBtk, with an 83% overall survival rate and a 92% response rate in CLL (Burger, 2019). However, second generation Btk inhibitors were deemed necessary due to the adverse side effects of ibrutinib. These side effects include thrombocytopenia, neutropenia, frequent severe infections, and bleeding events (Burger, 2019). One study found that the discontinuation rate of ibrutinib was 52.5% due to toxicities, the main causes of which were bleeding, infections and atrial fibrillation (Mato *et al.*, 2018). The side effects of ibrutinib are due to two factors: 1) Btk is expressed in other non-B cells including platelets; and 2) ibrutinib has off-target effects on other kinases.

Ibrutinib binds to the ATP-binding region of the Tec Kinase (TK) domain of Btk at a cysteine at position 481 to inhibit Btk (Honigberg *et al.*, 2010) with an IC_{50} *in vitro* of 0.5nM (Honigberg *et al.*, 2010). In terms of off-target effects, ibrutinib has an IC_{50} of 10, 96, 171 and 200nM for Tec, Fyn, Src and Lyn respectively (Honigberg *et al.*, 2010) (Barf *et al.*, 2017), all of which are expressed in platelets.

Second generation iBtks were designed for reduced toxicity and increased efficacity, by virtue of fewer off-target effects. These second-generation inhibitors include acalabrutinib. All second generation iBtks have reduced anti-haemostatic activity in comparison to ibrutinib; acalabrutinib has minimal off-target effects (Barf *et al.*, 2017). Acalabrutinib has a higher IC_{50} at 5nM for Btk inhibition but also a higher selectivity for Btk than ibrutinib. Acalabrutinib has an IC_{50} for Tec at 126nM though it does not inhibit Fyn, Src and Lyn (Barf *et al.*, 2017).

Btk is expressed in other cells, such as myeloid lineage cells, and as such Btk inhibitors may have an effect in these cells. Btk is found in platelets downstream of the ITAM receptors GPVI and FcγRIIa. Tec is also found in platelets and has been previously demonstrated to substitute for Btk in the GPVI pathway (Atkinson, Ellmeier and Watson, 2003b). Btk inhibition by ibrutinib in platelets has an anti-haemostatic effect. Ibrutinib-treated patients may have mild to life threatening bleeding phenotypes owing to the effects of ibrutinib on the platelet GPVI pathway. A bleeding phenotype is also seen in some patients treated with acalabrutinib, however, this tends to be milder than that observed in ibrutinib-treated patients (Series *et al.*, 2019). Other studies have found that ibrutinib can inhibit Btk downstream of GPVI signalling, as well as Tec kinase (Nicolson *et al.*, 2018a). Other platelet proteins, such as Src, have been seen to be effected by ibrutinib(Bye *et al.*, 2017).

The bleeding phenotype displayed in ibrutinib-treated patients correlates to the observed reduction in collagen stimulated platelet aggregation (Kamel *et al.*, 2015) (Levade *et al.*, 2014) (Lipsky *et al.*, 2015) implicating the GPVI receptor pathway in ibrutinib inhibition. Platelets from ibrutinib-treated patients in *ex vivo* studies had a reduction in GPVI signalling. In the same *ex vivo* studies, acalabrutinib-treated CLL patient platelets did not show a reduction in GPVI signalling (Nicolson *et al.*, 2018a). Another difference between ibrutinib and acalabrutinib lies in off-target effects on Src and Tec. Ibrutinib can inhibit Src and Tec whilst acalabrutinib is more specific for Btk and as such thrombus formation under arterial flow is not inhibited in response to collagen (Bye *et al.*, 2017).

αIIbβ3 mediated platelet adhesion to fibrinogen is affected by ibrutinib due to inhibition of outside-in signalling of αIIbβ3. This results in reductions in platelet spreading and clot retraction (Bye *et al.*, 2015a). αIIbβ3 deficient mice experience rebleeding in tail bleed experiments suggesting that αIIbβ3 outside-in signalling inhibition by ibrutinib could contribute towards the bleeding phenotype observed in ibrutinib-treated patients (Bye *et al.*, 2015a). Moreover, adhesion to VWF is decreased in ibrutinib-treated patients and in healthy control platelets incubated *in vitro* with ibrutinib which overall would lead to a diminished ability for thrombus formation (Levade *et al.*, 2014).

In B-cell malignancies, ibrutinib treatment is associated with an increased risk of infections. Platelets can be activated in response to bacteria. This activation can result in scavenging of bacteria, release of antimicrobial substances, direct interaction with leukocytes (Ali, Wuescher and Worth, 2015) (Gaertner *et al.*, 2017) (Palankar *et al.*, 2018). However, the potential effects of ibrutinib and acalabrutinib on platelet immune function have not been evaluated.

A pathway of importance in platelet-bacteria interactions is IgG-opsonised bacteria binding the platelet low affinity IgG immune receptor, FcγRIIa. Signalling through FcγRIIa activates Src-family kinases, in turn activating Syk. This results in the activation of Btk, LAT and SLP76, causing the phosphorylation and activation of PLCγ2. However, the role of Btk in FcγRIIa signalling has previously only been studied by crosslinking FcγRIIa with antibody, which utilises different signalling than when FcγRIIa is stimulated with bacteria; the latter requires αIIbβ3, ADP and TXA_2 (Arman *et al.*, 2014). As such, the effects of FcγRIIa activation on Btk by bacteria are unknown. Responses in platelets seen to be mediated through the FcγRIIa receptor include: aggregation and immunothrombosis, granule release, internalisation of IgG immune complexes and bacterial killing (Arman and Krauel, 2015). The interactions between platelets and bacteria can be aided by components in the plasma, such as immunoglobulins. This could indicate that ibrutinib and acalabrutinib reduce platelet-mediated immune responses seen when platelets interact with bacteria.

Hypothesis

The hypothesis of the chapter is: iBtks impair human platelet responses to bacteria by the inhibition of FcγRIIa function.

Aim of this chapter

The main question which will be addressed in this chapter is:

- Do iBtks ibrutinib and acalabrutinib impair FcγRIIa-mediated platelet responses to bacteria in healthy donor platelets?

To answer this question, isolated platelets were freshly prepared from healthy donors in the presence or absence of plasma (PRP or washed platelets respectively). Platelet preparations were incubated *in vitro* with varying doses of ibrutinib and acalabrutinib,

followed by agonists stimulation. Platelet activation was measured as platelet aggregation (LTA assay), α-granule secretion (PF4 ELISA), and dense granule release (ATP luciferin-luciferase assay). It was important to begin the study with healthy controls rather than CLL platelets to exclude other potential CLL-specific factors that might affect platelet responses to bacteria.

The anti-FcγRIIa mAb, IV.3, was crosslinked to cause direct clustering of the FcγRIIa receptor thus inducing FcγRIIa-mediated platelet activation. In addition, this study utilised Gram-positive *S. aureus* Newman and Gram-negative *E. coli* RS218 bacteria. Despite having different cell surface molecular compositions, both strains cause FcγRIIa-mediated platelet activation (Hawiger *et al.*, 1979) (Watson *et al.*, 2016). The TRAP6 amide peptide that activates platelets through the thrombin/PAR1 signalling pathway was used as a negative control as Btk is not activated in this pathway (Ninomoto *et al.*, 2020) (Bye *et al.*, 2017) (Series *et al.*, 2019). CRP-XL was used to directly activate GPVI receptors (Morton *et al.*, 1995).

3.2 Results

3.2.1 Ibrutinib and acalabrutinib inhibit GPVI-mediated platelet aggregation in washed platelets and PRP

Previous studies have demonstrated that ibrutinib and acalabrutinib inhibit the GPVI pathway in platelets in response to collagen and CRP as assessed by LTA. To ensure these published results were reproducible in a local healthy donor cohort, aggregation was first performed with the GPVI specific agonist CRP upon platelet incubation with ibrutinib but also acalabrutinib (Figure 3.1 and 3.2). Results show inhibitory effects of the two iBtks in the present study that were in line with previously published inhibitory doses of these drugs in washed platelets (Nicolson *et al.*, 2018a).

In both Figure 3.1 A with 3µg/ml CRP and Figure 3.2 A with 1µg/ml CRP, washed platelet aggregation is inhibited at a lower dose of ibrutinib than that required for PRP, Figure 3.1 B and Figure 3.2 B respectively. A slight dose dependency can be seen with ibrutinib and PRP for 3µg/ml CRP (Figure 3.1 B), while this is not evident with acalabrutinib (Figure 3.1 D).

The lack of strong ibrutinib inhibition of CRP-induced aggregation in PRP is similar to studies by others, in which high concentrations of ibrutinib were needed to inhibit GPVI signalling (Nicolson 2018). When a higher concentration of CRP is used, a greater concentration of ibrutinib was required to inhibit aggregation in PRP, e.g., 5µM ibrutinib only impaired 3µg/ml CRP-induced aggregation but completely blocked aggregation induced by 1µg/ml CRP. This effect was not seen in washed platelets. PRP reactions also required a higher concentration of iBtk to inhibit aggregation compared to washed platelets, which could be due to the absence of plasma proteins in washed platelets. The concentration of acalabrutinib used in this study had no significant inhibition upon CRP stimulation of PRP.

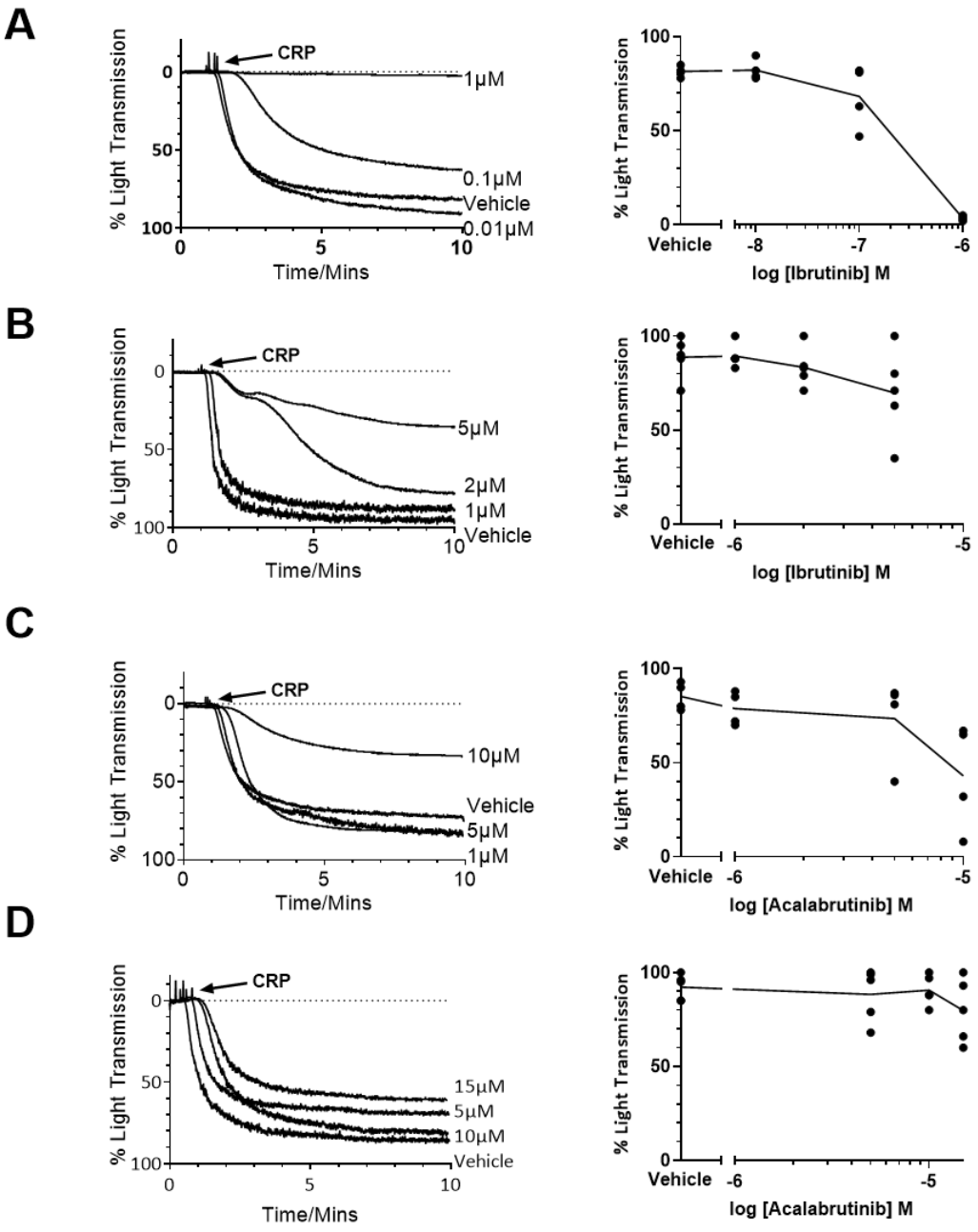


Figure 3.1 iBtks, ibrutinib and acalabrutinib, affect the platelet GPVI activation pathway to 3μg/ml CRP in washed platelets and PRP. The effect of iBtks on GPVI-mediated platelet aggregation was determined by incubation of platelets with iBtks for 5 minutes before the addition of 3μg/ml CRP and measured by LTA. The effect of ibrutinib on GPVI pathway in (A) washed platelets and (B) PRP. The effect of acalabrutinib on GPVI pathway in (C) washed platelets and (D) PRP. PRP at concentrations between 2.3-5.7x10^8 platelets/ml. Washed platelets at concentration of 2.5x10^8 platelets/ml.

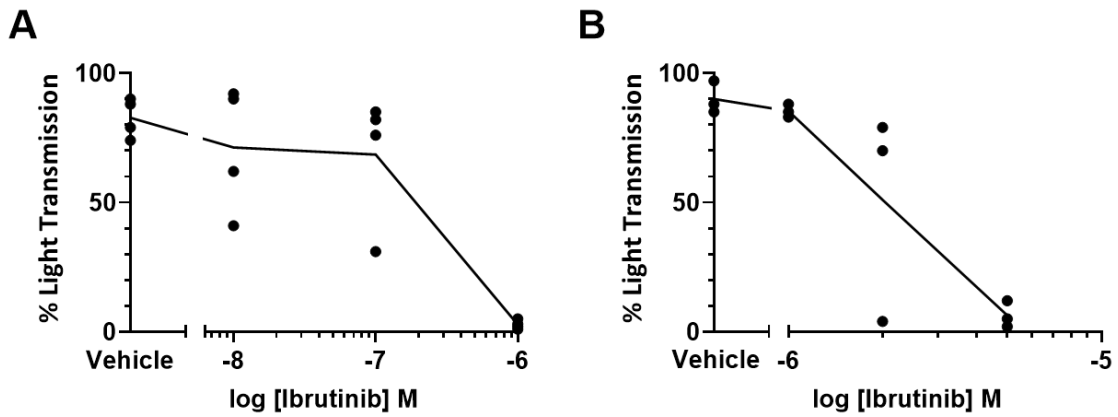


Figure 3.2 Ibrutinib affects the platelet GPVI activation pathway to 1µg/ml CRP in washed platelets and PRP. The effect of ibrutinib on GPVI-mediated platelet aggregation was determined by incubation of platelets with ibrutinib for 5 minutes before the addition of 1µg/ml CRP and measured by LTA for 10 minutes. The effect of ibrutinib on GPVI pathway in (A) washed platelets and (B) PRP. PRP at concentrations between 2.3-5.7x10^8 platelets/ml. Washed platelets at concentration of 2.5x10^8 platelets/ml.

3.2.2 Ibrutinib and acalabrutinib dose-dependently inhibit platelet aggregation in response to FcγRIIa agonists in washed platelets

To establish the effects of ibrutinib and acalabrutinib on platelet FcγRIIa-mediated aggregation, washed platelets were incubated with these iBtks separately. Samples were stimulated by known FcγRIIa agonists: crosslinked IV.3 mAb (IV.3 XL) and bacteria. IV.3 XL directly activates FcγRIIa and causes immediate platelet aggregation. In contrast, *S. aureus* Newman and *E. coli* RS218 are known to act through FcγRIIa but aggregation takes place after a few minutes of platelet stimulation (usually within the first 20 minutes of incubation), which is known as lag time.

A large number of bacteria have been studied and aggregation as a response to those bacteria is described as an all-or-nothing response as after lag time, complete aggregation usually occurs (Arman *et al.*, 2014) (Watson *et al.*, 2016). This differs from conventional agonists which, after the short or undetectable lag time, can result in partial aggregation depending on the concentration of agonist used. Lag time is theorised as a result of weaker platelet receptor activation, and/or a longer time for the platelet receptor to bind to the bacteria (Hamzeh-Cognasse *et al.*, 2015). *S. aureus* and *E. coli* were chosen to be the primary bacteria used in these studies due to their relevance in CLL (Samet *et al.*, 2013) (Andersen *et al.*, 2019) (Korona-Glowniak *et al.*, 2019), being common causes of bacterial infection in patients, as well as representing both Gram positive and negative bacteria, respectively.

LTA with platelets co-incubated with bacteria was recorded for 20 minutes. CFUs for both prepared bacteria suspensions were $1x10^9$ CFU/ml. TRAP6, a PAR1 agonist, was used as a negative control agonist.

A significant inhibition of IV.3 XL induced washed platelet aggregation was observed with 1µM ibrutinib, as well as with both strains of bacteria. Acalabrutinib also had a dose-dependent inhibitory effect on washed platelet aggregation in response to FcγRIIa ligands, with 10µM acalabrutinib causing significant inhibition. IC50s for ibrutinib and IV.3 XL, *S. aureus* Newman, and *E. coli* RS218 were 0.013, 0.313 and 0.0847µM respectively. For acalabrutinib, the IC50s for IV.3 XL, *S. aureus* Newman, and *E. coli* RS218 were 6.821, 7.336 and 4.798µM respectively.

Lag time was calculated from time of bacterial agonist addition to start of aggregation in all cases where aggregation was not inhibited by ibrutinib or acalabrutinib.

As previously reported, there was variability of lag times between individual donors in the case of *S. aureus* Newman stimulation. Therefore, to evaluate the effects of iBtks on this parameter, changes in lag time compared to vehicle (e.g., platelets from the same donor stimulated with bacteria in the absence of iBtk) were calculated (Figure 3.5). This analysis was not applicable to *E. coli* RS218 because both ibrutinib and acalabrutinib inhibited all reactions completely.

Platelets incubated with 0.1µM ibrutinib prior to stimulation with *S. aureus* Newman and showed aggregation had an increase in lag time for all but one donor with a mean increase lag time of 52.5 ±37.75 seconds (Figure 3.5 A). In the case of platelets incubated with acalabrutinib prior to stimulation with *S. aureus* Newman (Figure 3.5 B), lag time is increased for each donor in a dose-dependent fashion, as such 1µM acalabrutinib results in 60 ±48.89 seconds (n=4), 5µM acalabrutinib results in 165 ±30 seconds (n=4), and 10µM results in 180 ±0 seconds (n=2) increase in lag time. This suggest that ibrutinib is slowing down time to platelet activation in reactions where aggregation still takes place.

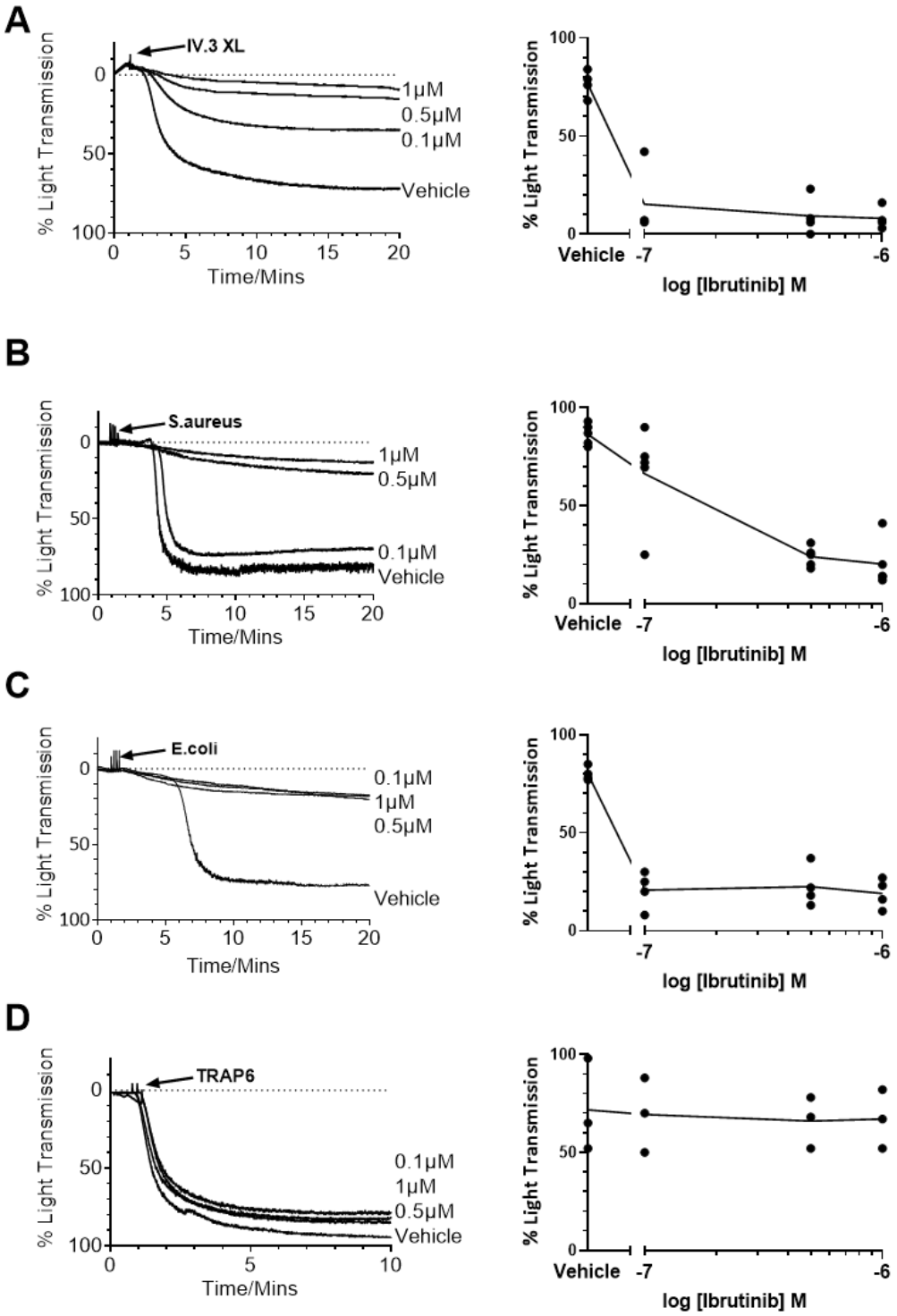


Figure 3.3 Ibrutinib inhibits platelet aggregation in response to crosslinked IV.3 mAb and bacteria in washed platelets. Washed platelets were incubated with ibrutinib for 5 minutes before the addition of (A) 4μg/ml mAb IV.3 for 2 minutes followed by 30μg/ml F(ab')$_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218, or (D) 3μM TRAP6. Platelet aggregation was measured by LTA. Washed platelets at concentration of 2.5x10^8 platelets/ml.

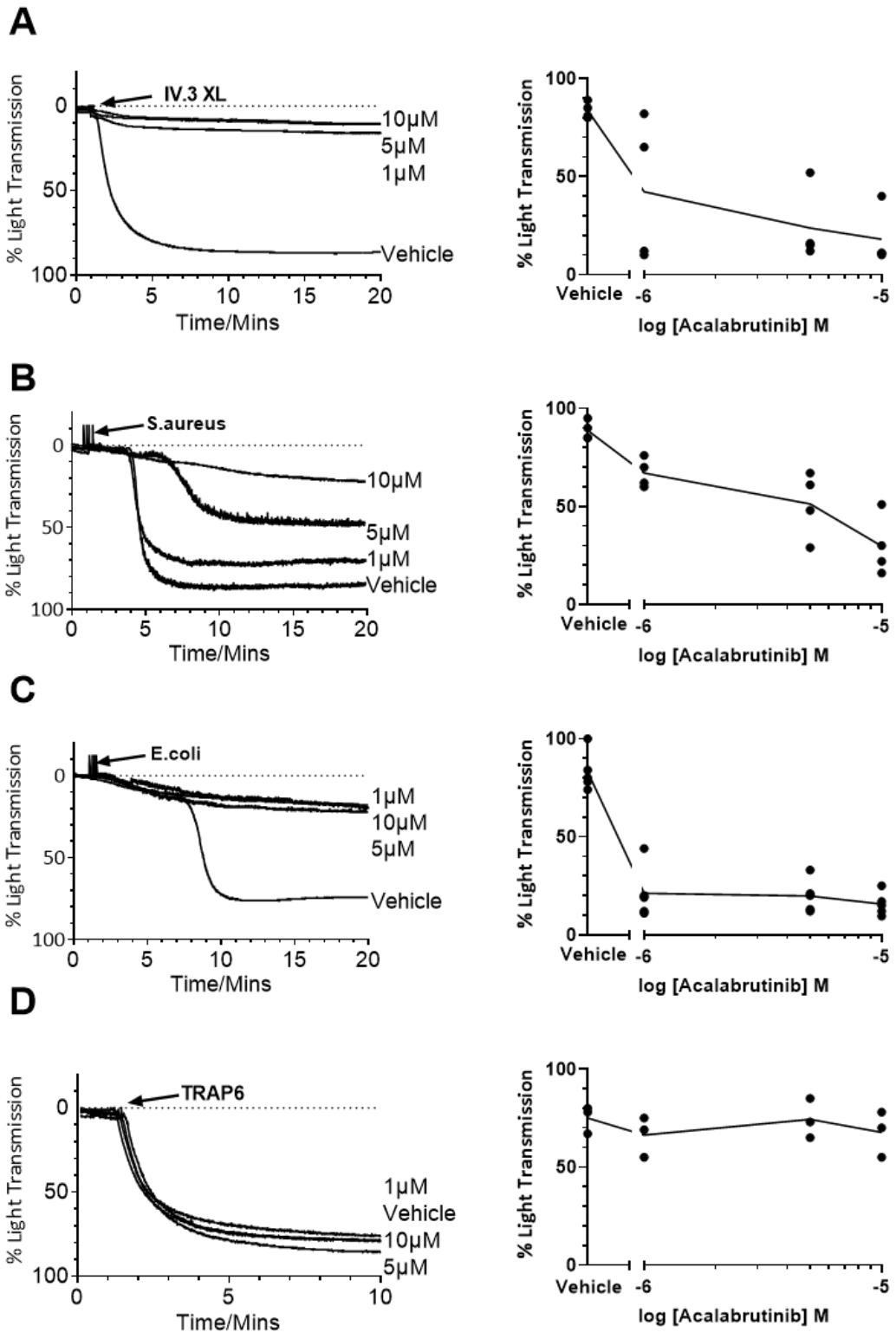


Figure 3.4 Acalabrutinib inhibits platelet aggregation in response to crosslinked IV.3 mAb and bacteria in washed platelets. Washed platelets were incubated with acalabrutinib was incubated with washed platelets for 5 minutes before the addition of (A) 4μg/ml mAb IV.3 for 2 minutes followed by 30μg/ml $F(ab')_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218, or (D) 3μM TRAP6. Platelet aggregation was measured by LTA. Washed platelets at concentration of $2.5x10^8$ platelets/ml.

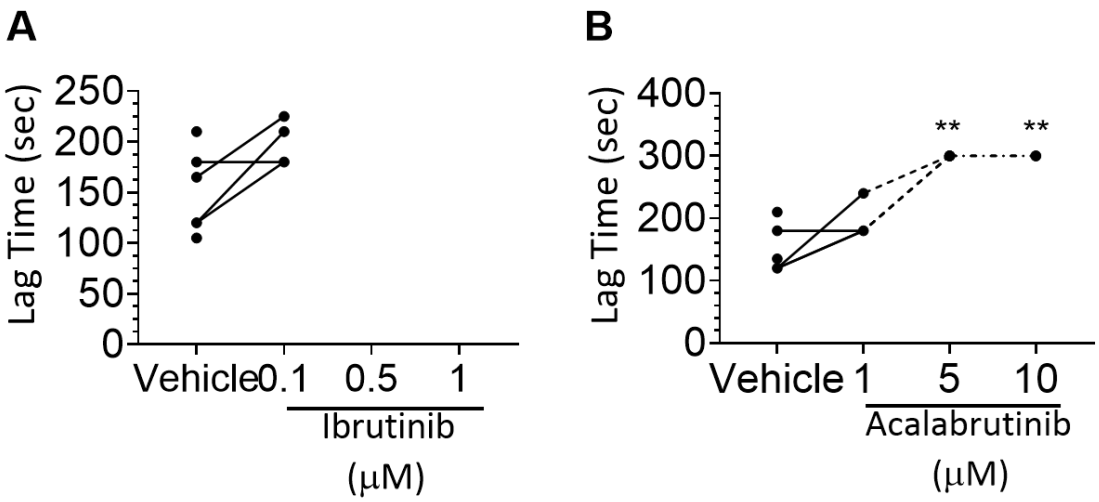


Figure 3.5 iBtks increase the lag time to platelet aggregation in response to S. aureus Newman in washed platelets. Data is linked to Figure 3.3 B and 3.4 B, showing lag times for all reactions in which there was no inhibition of aggregation. Washed platelets were incubated with (A) ibrutinib or (B) acalabrutinib for 5 minutes before the addition of *S. aureus* Newman. Washed platelets at concentration of $2.5x10^8$ platelets/ml. Lag time to aggregation was measured by LTA. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 in comparison to vehicle.

3.2.3 Ibrutinib and acalabrutinib dose-dependently inhibit platelet aggregation in response to bacteria in PRP

Ibrutinib and acalabrutinib are known to bind plasma proteins reversibly, mainly albumin, at 97.3% and 97.5% respectively (Bose, Gandhi and Keating, 2016) (Podoll *et al.*, 2019). As such, dose curves for each drug were performed with PRP to observe the effect the presence of plasma had on the inhibition of platelet aggregation to FcγRIIa agonists in comparison to the inhibition detected in washed platelets, with the former being more physiologically relevant. Additionally, bacteria are known to require components of the plasma, such as immunoglobulins, to interact with platelets, as well as other unidentified components (Fitzgerald, Foster and Cox, 2006). These components may change between individuals. PRP was incubated for 5 minutes with either ibrutinib or acalabrutinib before stimulation with either IV.3 XL, *S. aureus* Newman and *E. coli* RS218.

Platelet activation by all FcγRIIa agonists experienced dose-dependent inhibition by ibrutinib in PRP (Figure 3.6). IV.3 XL induced platelet aggregation appears more sensitive to ibrutinib than that observed upon bacteria stimulation. A higher concentration of ibrutinib is needed to inhibit aggregation in PRP to all FcγRIIa agonists tested when compared to washed platelets (Figure 3.6 and Figure 3.3 respectively).

A higher concentration of acalabrutinib was required to achieve complete inhibition of aggregation in PRP (Figure 3.7) in comparison to that used in washed platelets (Figure 3.4). This inhibition is dose dependent. As seen for ibrutinib, platelets incubated with acalabrutinib and then stimulated with IV.3 XL (Figure 3.7 A) experience inhibition at lower concentrations of acalabrutinib than those observed with bacteria, (Figure 3.7 B and C). IV.3 XL may create a stronger FcγRIIa response than bacteria, with the latter probably resulting from the combination of multiple mechanisms by which platelet aggregation is induced.

IC50s for ibrutinib and IV.3 XL, *S. aureus* Newman, and *E. coli* RS218 were 1.363, 2.391 and 2.82µM respectively. For acalabrutinib, the IC50s for IV.3 XL, *S. aureus* Newman, and *E. coli* RS218 were 8.711, 10.43 and 15.47µM respectively.

Lag time was measured from PRP LTA reactions as shown in Figure 3.8. A lack of data point for an individual corresponds to inhibition of aggregation and so no lag time calculated. In each donor it was observed that lag time was increased for each drug and bacteria combination in an iBtk dose-dependent manner, in that the higher the iBtk concentration the longer the lag time. This is like the effects previously seen in washed platelets with *S. aureus* Newman (Figure 3.5).

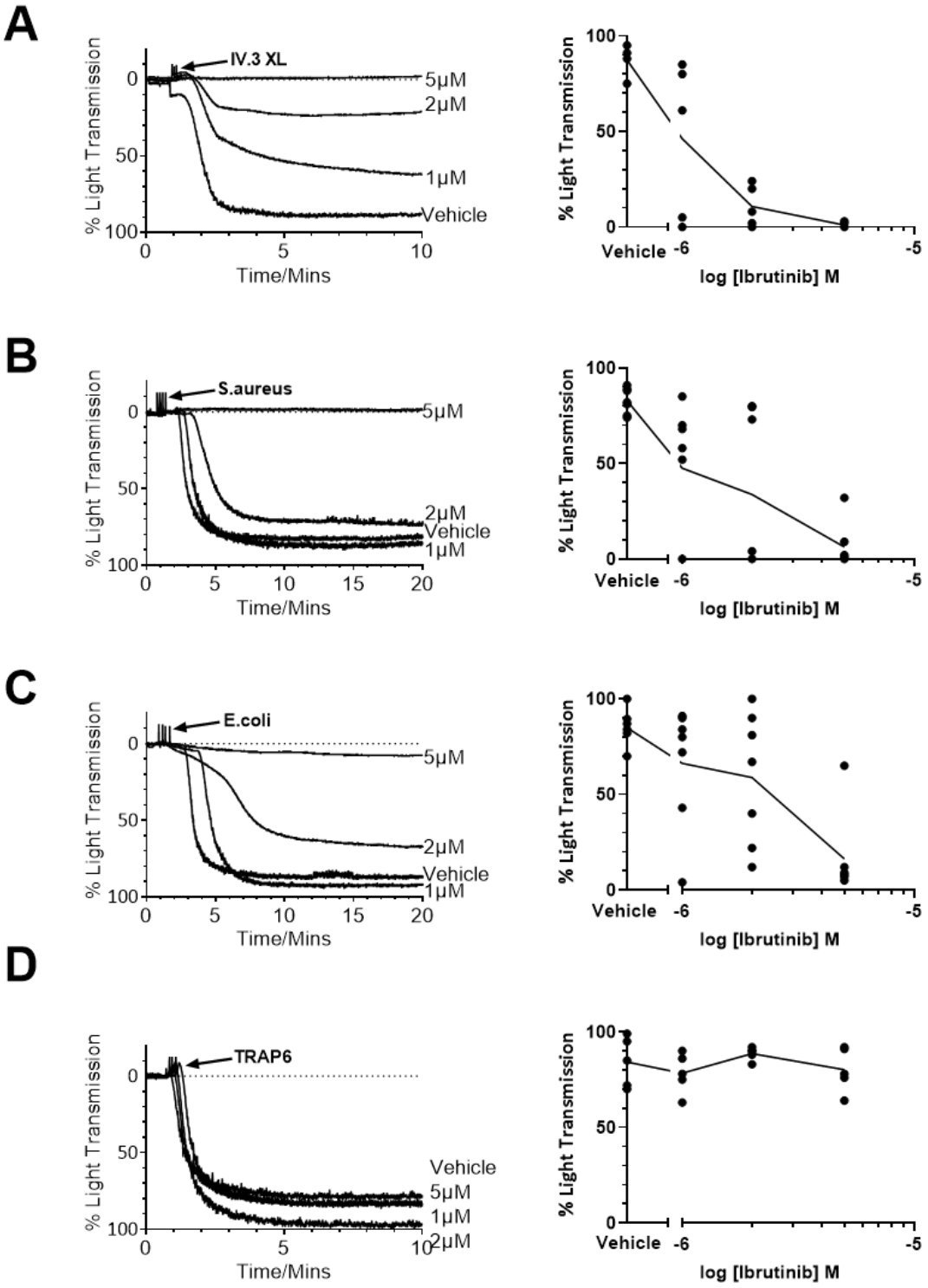


Figure 3.6 Ibrutinib inhibits platelet aggregation in response to FcγRIIa agonists in PRP. PRP at concentrations between 2.6-6.5x10^8 platelets/ml was incubated for 5 minutes with ibrutinib or vehicle before the addition of (A) 4μg/ml mAb IV.3 for 2 minutes followed by 30μg/ml $F(ab')_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218, or (D) 3μM TRAP6. Platelet aggregation was measured by LTA.

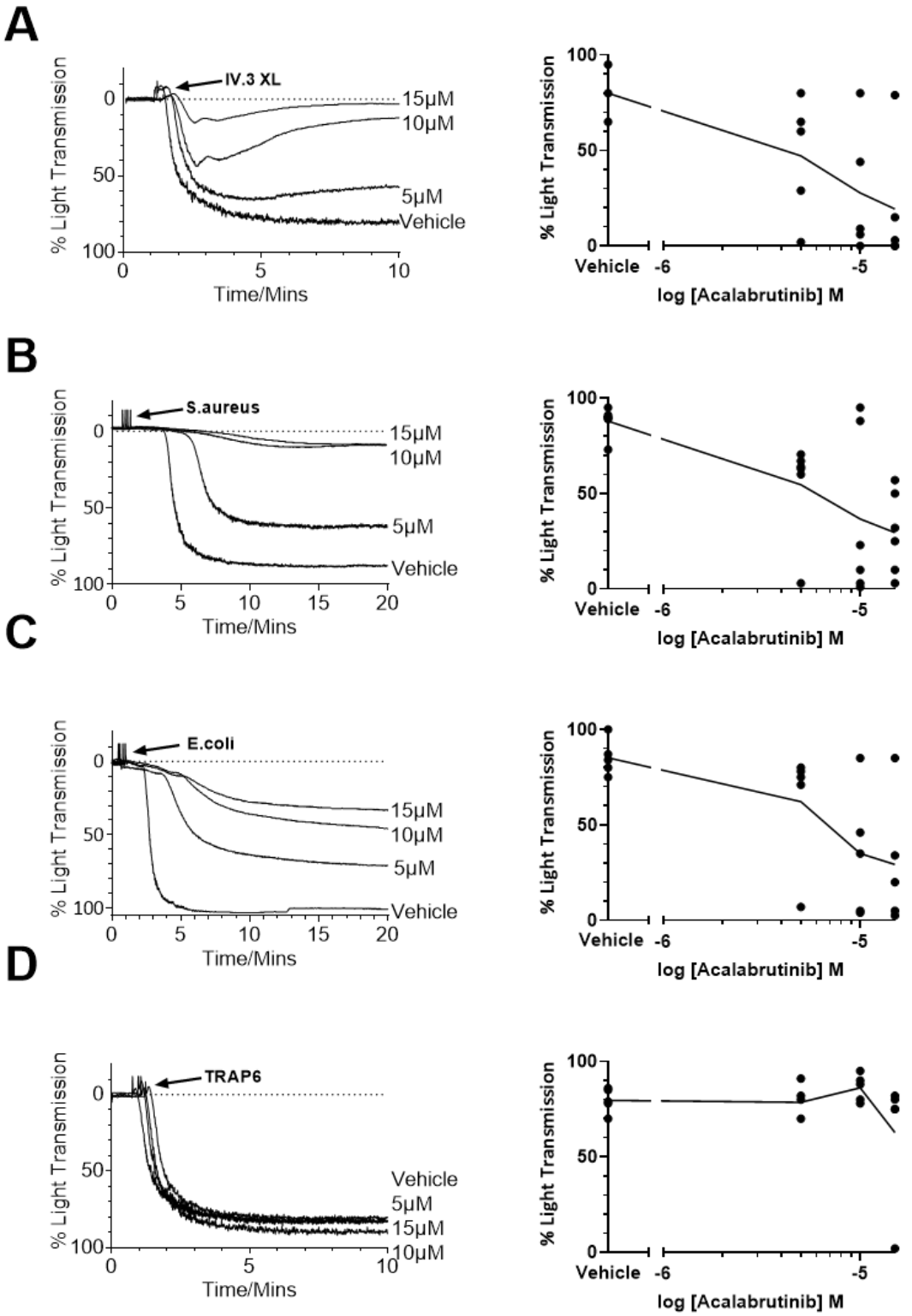


Figure 3.7 Acalabrutinib inhibits platelet aggregation in response to FcγRIIa agonists in PRP. PRP at concentrations between 2.6-6.5x10^8 platelets/ml was incubated for 5 minutes with acalabrutinib or vehicle before the addition of (A) 4μg/ml mAb IV.3 for 2 minutes followed by 30μg/ml $F(ab')_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218, or (D) 3μM TRAP6. Platelet aggregation was measured by LTA.

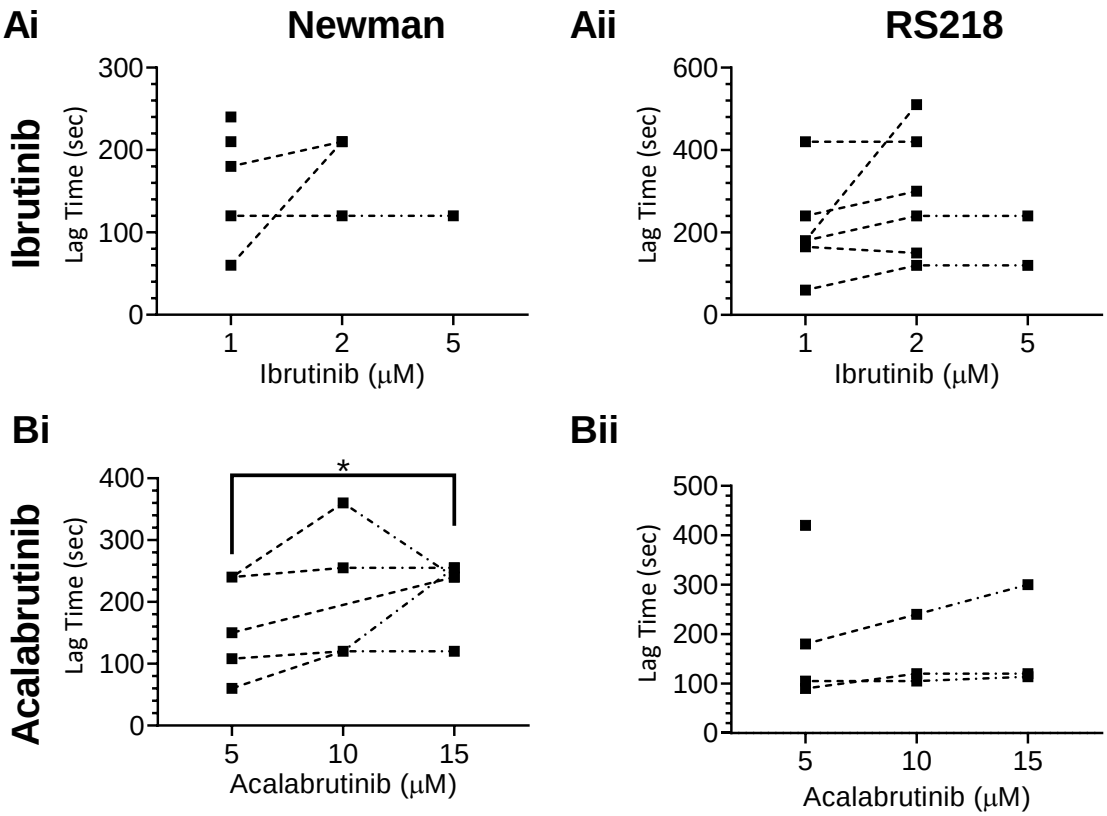


Figure 3.8 iBtks increase the lag time to platelet aggregation in response to bacteria in PRP. This data is linked to Figure 3.6 and 3.7, showing lag times for all reactions in which aggregation was not inhibited and calculated by LTA. PRP was incubated with (A) Ibrutinib or (B) acalabrutinib for 5 minutes before the addition of (i) *S. aureus* Newman or (ii) *E. coli* RS218. Lag time to aggregation was determined by LTA. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 in comparison to vehicle.

3.2.4 Inhibition of bacteria-induced aggregation by ibrutinib and acalabrutinib is not affected by increased iBtk incubation times.

To explore if the inhibition of the FcγRIIa pathway is dependent on the platelet incubation time with ibrutinib or acalabrutinib, PRP was incubated with varying doses of iBtk for either 5, 20 or 40 minutes before the addition of bacteria (Figure 3.9). For both ibrutinib and acalabrutinib, incubation time had no clear effect on final aggregation in response to either of the bacteria. Data presented in Figure 3.9 shows the same trend of iBtk dose curve inhibition of aggregation regardless of the drug incubation time tested, especially for *E. coli* RS218. This suggests that maximal inhibition by ibrutinib and acalabrutinib is reached quickly.

To investigate whether the increase in lag time seen with the use of ibrutinib and acalabrutinib with bacteria (Figure 3.10) is dependent on the length of platelet preincubation time with the drug, lag times were calculated from the bacteria-induced aggregation responses with PRP incubated with the iBtks for 5, 20 or 40 minutes. In Figure 3.10, no change in lag time is seen through increasing the concentration of each iBtk through each of the time points.

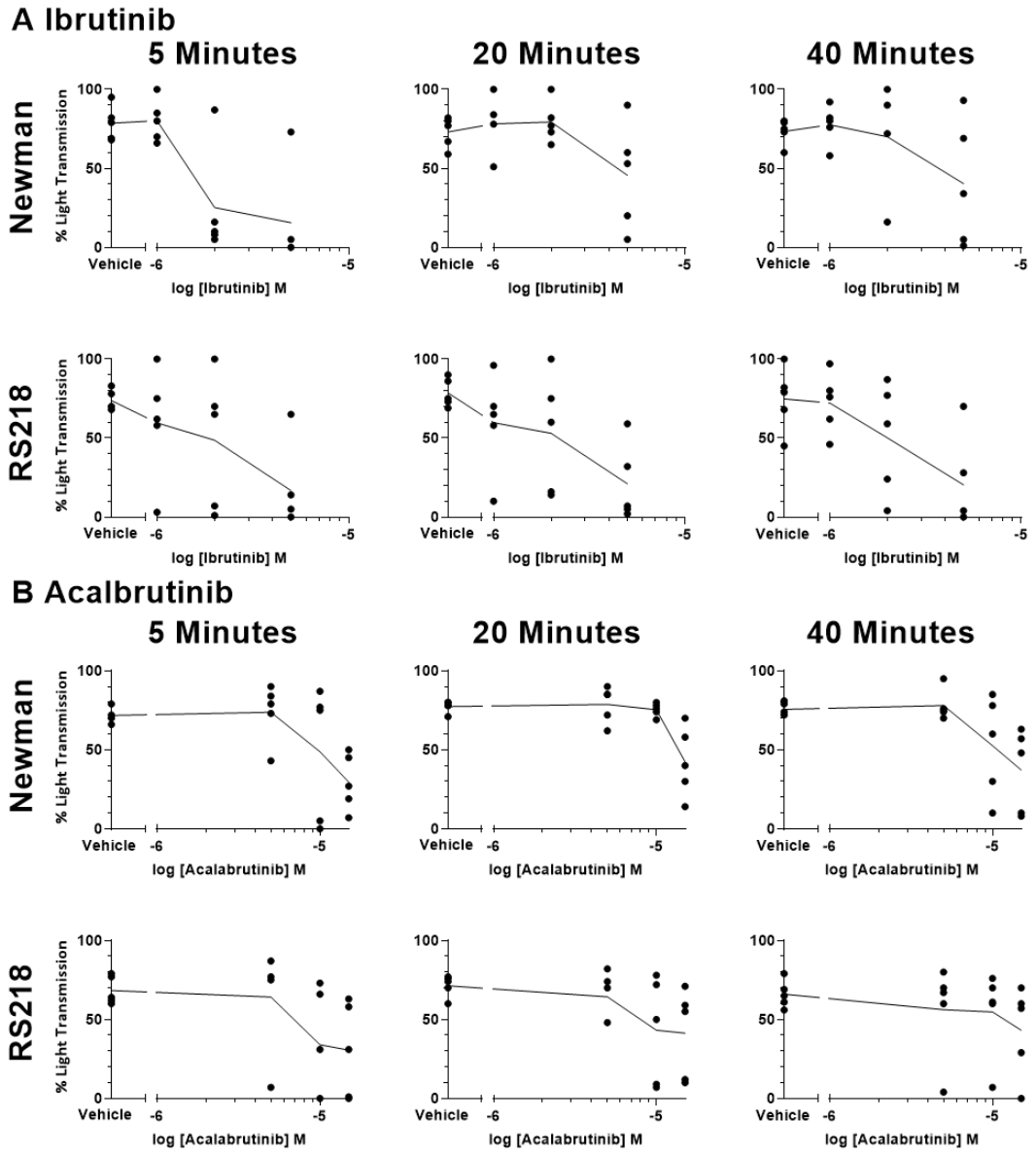


Figure 3.9 Length of iBtk incubation has no effect on final platelet aggregation in response to bacteria. PRP at concentrations between 3.4-5.1x10^8 platelets/ml was incubated with (A) ibrutinib or (B) acalabrutinib for 5, 20 or 40 minutes before the addition of *S. aureus* Newman or *E. coli* RS218. Aggregation was measured by LTA. Graphs show maximum aggregation.

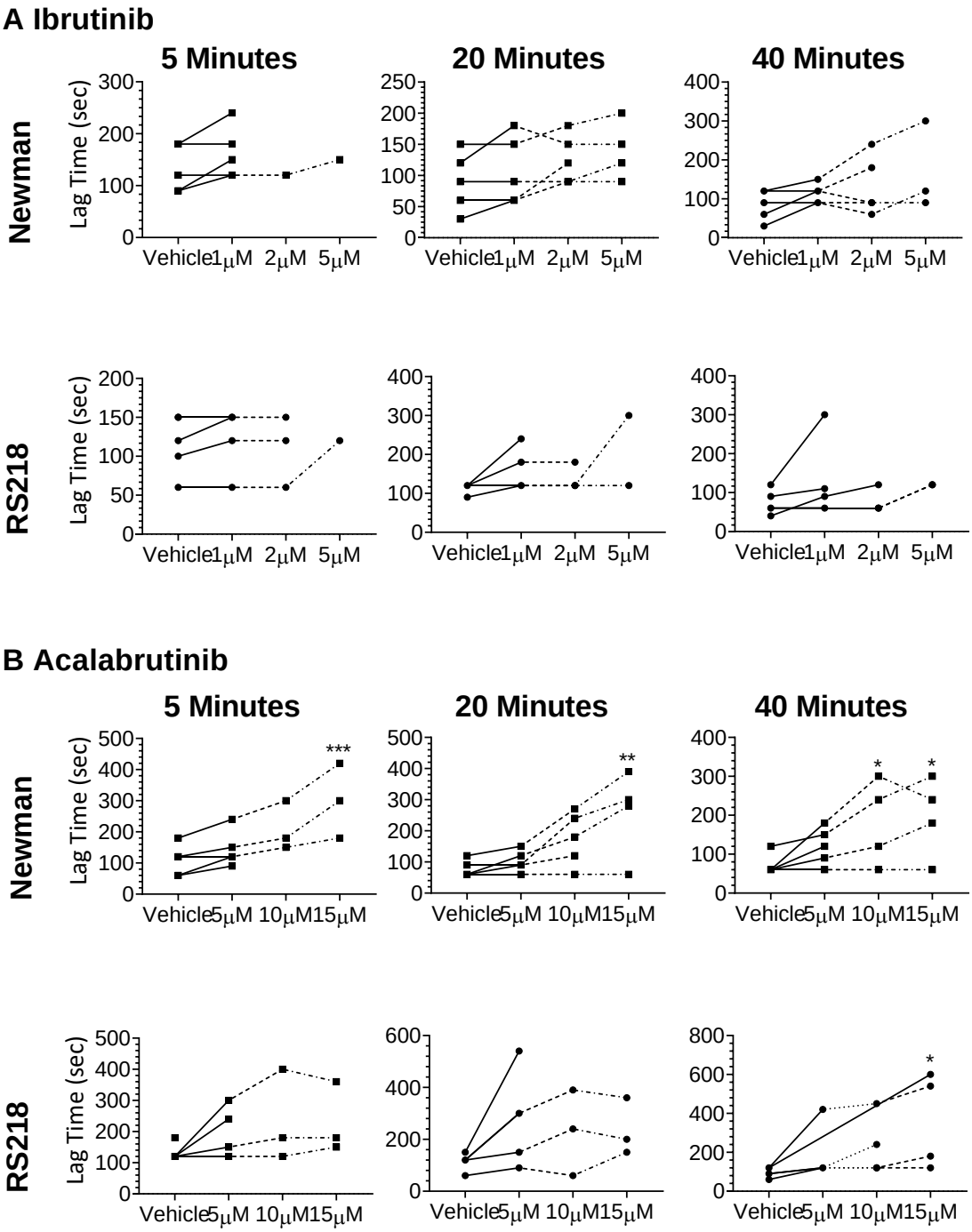


Figure 3.10 Length of iBtk incubation has no effect on lag time to platelet aggregation in response to bacteria. Figure linked to Figure 3.9 showing lag times for all reactions in which there was no inhibition of aggregation. Change of lag time was calculated by comparison to vehicle reaction. PRP was incubated with (A) ibrutinib or (B) acalabrutinib for 5, 20 or 40 minutes before the addition of *S. aureus* Newman or *E. coli* RS218. The lag time to aggregation was determined by LTA. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 in comparison to vehicle.

3.2.5 Ibrutinib and acalabrutinib inhibit bacteria-induced platelet aggregation that is dependent on the FcγRIIa receptor pathway

Bacteria can interact with platelets in numerous molecular ways, with some of these interactions resulting in platelet aggregation. Despite some bacteria-strain specific molecular interactions, the FcγRIIa signalling is needed to trigger aggregation for many strains of bacteria. To ensure that the bacteria used in this study caused aggregation in a manner observed by others (Arman *et al.*, 2014) (Arman and Krauel, 2015) (Watson *et al.*, 2016), LTA was performed with PRP in the presence of specific inhibitors (Figure 3.11). FcγRIIa-dependent platelet activation in response to bacteria (but not for receptor-antibody crosslinking) requires activity of receptors FcγRIIa and αIIbβ3 (as evidenced by the lack of platelet secretion in the presence of αIIbβ3 blockers (Arman *et al.*, 2014)). Moreover, protein kinases, Src and Syk, are key components of the FcγRIIa intracellular signalling cascade. As such IV.3. mAb (FcγRIIa inhibitor), eptifibatide (αIIbβ3 inhibitor), dasatinib (Src inhibitor) and PRT-318 (Syk inhibitor) were used to inhibit these pathway proteins respectively. Ibrutinib and acalabrutinib were used at concentrations determined in section 3.2.3. After PRP incubation with inhibitors, platelets were stimulated with either IV.3 XL, *S. aureus* Newman or *E. coli* RS218 (Figure 3.11).

Figure 3.11 demonstrates that all agonists tested, except TRAP6 amide, activate platelets through FcγRIIa, as evidenced by significant inhibition of aggregation by IV.3 mAb, with P values of <0.001 and 0.004 for *S. aureus* Newman and *E. coli* RS218 respectively. In the case of FcγRIIa agonists, all inhibitors tested result in significant decreases of aggregation compared to the vehicle. For IV.3 XL and *S. aureus* Newman (Figure 3.11 A and B) P vales for dasatinib, PRT-318, ibrutinib, acalabrutinib, and eptifibatide are all <0.001, except for *S. aureus* Newman and acalabrutinib which is 0.02. For *E. coli* RS218 (Figure 3.11 C) P values for dasatinib, PRT-318, ibrutinib, acalabrutinib, and eptifibatide are 0.002, <0.001, 0.002, 0.001 and 0.001 respectively. iBtks also inhibited aggregation to IV.3 XL and bacteria as shown previously in section 3.2.3, and at levels comparable to Src, Syk, αIIbβ3, and FcγRIIa inhibition.

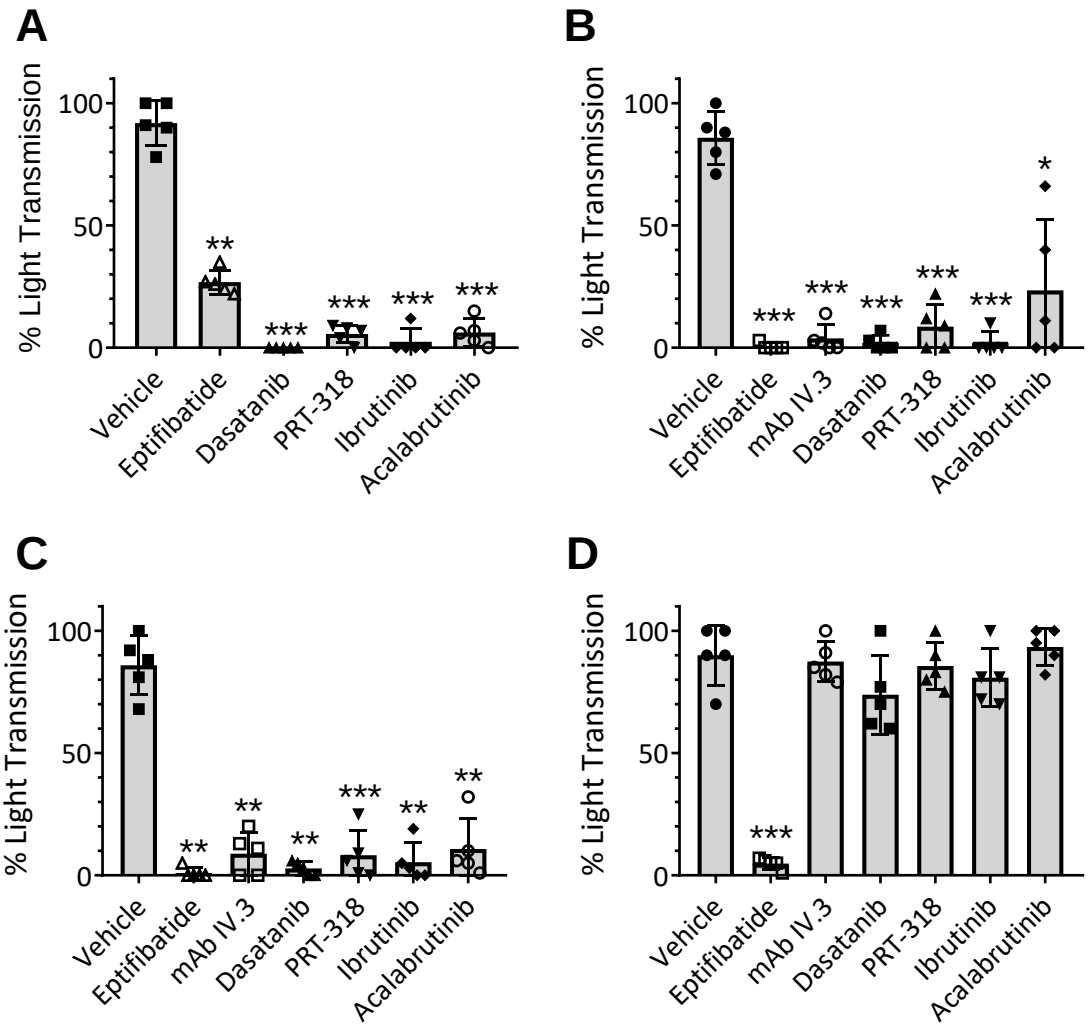


Figure 3.11 Bacteria cause FcγRIIa-mediated platelet aggregation that is inhibited by FcγRIIa, αIIbβ3, Src, Syk and Btk inhibitors. PRP at concentrations between 2.8-4.7x10^8 platelets/ml was incubated with either 9μM eptifibatide, 20μg/ml mAb IV.3, 4μM dasatinib, 10μM PRT-318, 5μM ibrutinib or 15μM acalabrutinib before stimulation by (A) 4μg/ml mAb IV.3 for 2 minutes followed by 30μg/ml F(ab')$_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218, or (D) 3μM TRAP6 amide. Graphs show maximum aggregation. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle.

3.2.6 Ibrutinib and acalabrutinib exert inhibitory effects on platelet aggregation to a range of bacteria

Ibrutinib and acalabrutinib inhibit FcγRIIa-dependent platelet aggregation in response to *S. aureus* Newman and *E. coli* RS218 as shown in section 3.2.5. To investigate if inhibition by ibrutinib and acalabrutinib would be reproducible with multiple bacteria, PRP was incubated with the maximum inhibitory dose of either ibrutinib or acalabrutinib before the addition of different bacterial strains known to cause platelet aggregation in an FcγRIIa-dependent manner: *S. aureus* Newman, *E. coli* RS218, *Streptococcus oralis* CR834, *Streptococcus sanguinis* 133-79, or *Streptococcus gordonii* DL1.

Ibrutinib and acalabrutinib both inhibit aggregation in response to all the bacteria tested in at least two out of three platelet donors tested (Figure 3.12). Ibrutinib gives P values of 0.001, 0.05, 0.02, 0.02 and 0.04 for *S. aureus* Newman, *E. coli* RS218, *S. sanguinis* 133-79, *S. gordonii* DL1, and *S. oralis* CR834 respectively. Acalabrutinib gives P values of 0.05, 0.09, 0.12, 0.01 and 0.04 for *S. aureus* Newman, *E. coli* RS218, *S. sanguinis* 133-79, *S. gordonii* DL1, and *S. oralis* CR834 respectively. The lack of statistically significant differences is due to the platelets of the same donor aggregating in response to the bacteria despite the addition of iBtks.

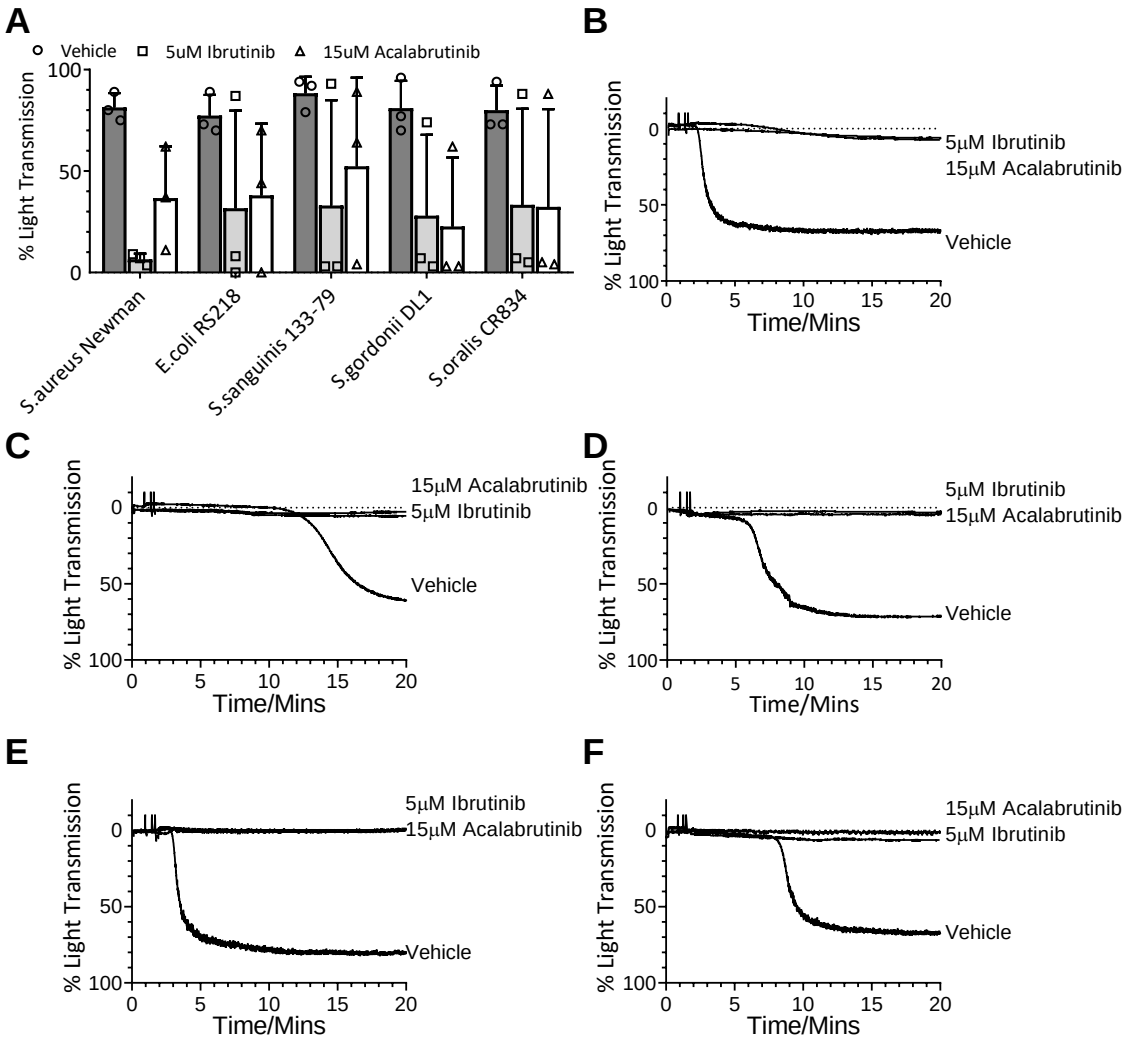


Figure 3.12 Ibrutinib and acalabrutinib inhibit platelet aggregation in response to a range of bacterial species. PRP at concentrations between 4.9-7.7x10^8 platelets/ml. (A) represents mean data for iBtk or vehicle treated PRP stimulated with (B) *S. aureus* Newman, (C) *Streptococcus oralis* CR834, (D) *Streptococcus sanguinis* 133-79, (E) *E. coli* RS218 or (F) *Streptococcus gordonii* DL1 for 20 minutes. Prior to agonist addition, platelets were pre-incubated with vehicle, acalabrutinib, or ibrutinib for 5 minutes. Graph shows maximum aggregation, data shown as mean +SD. Data shown in B-F is representative of n=3.

3.2.7 Ibrutinib and acalabrutinib inhibit FcγRIIa-dependent platelet dense granule secretion

To investigate if iBtks, ibrutinib and acalabrutinib, inhibit dense granule secretion from platelets in response to FcγRIIa agonists further aggregation reactions were performed (Figure 3.13). Five minutes after the start of aggregation, or the equivalent time point in cases of inhibition, a luciferin-luciferase assay was carried out. ATP release was used as a marker of dense granule release. 12µM TRAP6 amide, known to fully degranulate platelets, was used to calculate the 100% ATP release.

Figure 3.13 shows that dense granule release occurred in response to (A) IV.3 XL, (B) *S. aureus* Newman and (C) *E. coli* RS218. Levels of ATP release were similar for all agonists used, including TRAP6 amide, demonstrating the ability of bacteria to induce dense granule release. FcγRIIa inhibitors inhibited dense granule release, except in all TRAP6 amide reactions, as well as eptifibatide with IV.3 XL stimulated platelets as αIIbβ3, which is inhibited by eptifibatide, is not required for FcγRIIa signalling in this case. ATP release in response to IV.3 XL, *S. aureus* Newman and *E. coli* RS218 was significantly inhibited by ibrutinib and acalabrutinib with P values of <0.001 for iBtks.

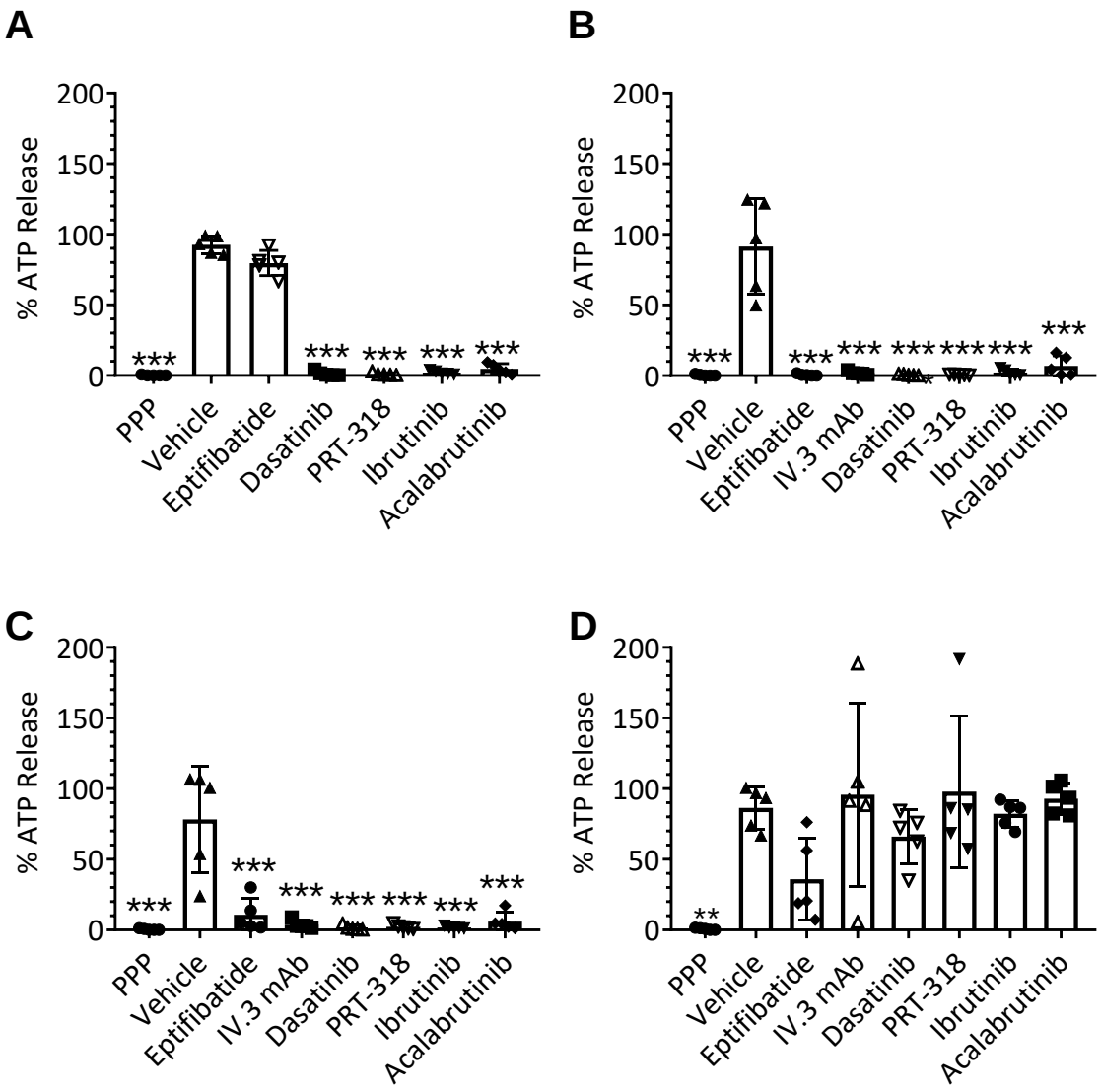


Figure 3.13 Platelet dense granule release in response to bacteria is mediated by the FcγRIIa pathway and is inhibited by ibrutinib and acalabrutinib. PRP at concentrations between 2.6-5.4x10^8 platelets/ml was incubated with assorted inhibitors: 9μM eptifibatide, 20μg/ml mAb IV.3, 4μM dasatinib, 10μM PRT-318, 5μM ibrutinib or 15μM acalabrutinib before stimulation by agonist. Supernatants were collected 5 minutes after platelet activation was seen or at the same time point in the case of inhibition, and a luciferin-luciferase assay was performed to measure ATP release. ATP release by 12μM TRAP6 amide was considered 100% release and used to normalise data for (A) 4μg/ml mAb IV.3 for 2 minutes followed by 30μg/ml $F(ab')_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218, or (D) 3μM TRAP6 amide. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle.

3.2.8 Ibrutinib and acalabrutinib inhibit FcγRIIa-dependent platelet α-granule secretion

The aim of this section was to determine if ibrutinib and acalabrutinib were able to inhibit α-granule secretion in platelets in response to bacteria, and if such inhibition targeted the FcγRIIa pathway. Aggregation reactions as seen in Figure 3.11 were performed, at the end of which prostacyclin was added before samples were centrifuged and the supernatant used for a PF4 ELISA, PF4 being a marker of α-granule release. PPP was used as a negative control due to the lack of platelets to release PF4.

Figure 3.14 shows that the levels of PF4 released in response to bacteria are similar to IV.3 XL and TRAP6 amide. Moreover, PF4 release in response to IV.3 XL and bacteria can be blocked by FcγRIIa pathway inhibitors (e.g., IV.3 mAb for bacteria, dasatinib and PRT-318 for both bacteria and IV.3 XL). Eptifibatide inhibited PF4 release from platelets stimulated with bacteria, but not IV.3 XL. This is due to αIIbβ3, which is inhibited by eptifibatide, not being required for platelet activation by IV.3 XL, while αIIbβ3 is required for platelet activation by bacteria through FcγRIIa.

Ibrutinib and acalabrutinib have similar significant inhibitory effects on α-granule release as observed with inhibition by IV.3 mAb, eptifibatide, dasatinib and PRT-318. Use of ibrutinib and acalabrutinib in Figure 3.14 result in P values of <0.001 in both cases for (A) IV.3 XL, <0.001 and 0.007 respectively for (B) *S. aureus* Newman, and <0.001 in both cases for (C) *E. coli* RS218. As such, it can be concluded that α-granule release in response to *S. aureus* Newman and *E. coli* RS218 is dependent on FcγRIIa and iBtks inhibit this process.

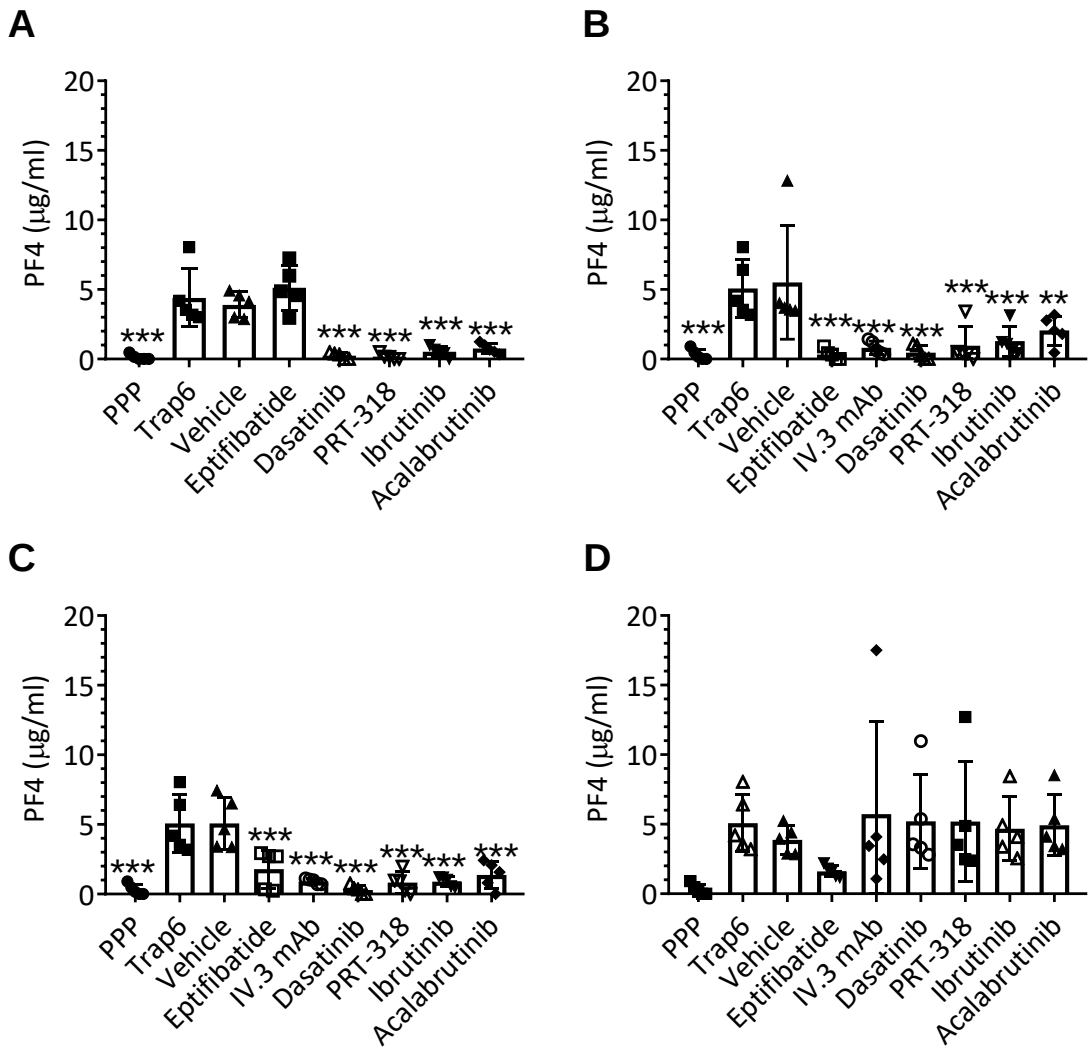


Figure 3.14 Platelet α-granule release in response to bacteria is mediated by the FcγRIIa pathway and is inhibited by ibrutinib and acalabrutinib. PRP at concentrations between 2.9-6.2x10^8 platelets/ml was incubated with assorted inhibitors including: 20µg/ml mAb IV.3, 5µM ibrutinib, 15µM acalabrutinib, 4µM dasatinib, 10µM PRT-318, 9µM eptifibatide before stimulation by agonist. Samples were collected 20 minutes after the addition of (A) 4µg/ml mAb IV.3 for 2 minutes followed by 30µg/ml $F(ab')_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218, or 10 minutes after the addition of (D) 3µM TRAP6. In A-C 12µM TRAP6 amide is included as a control for α-granule release. Supernatants were collected by centrifuging samples at 1500g with 4µM prostacyclin at room temperature, which were then measured for PF4 via ELISA. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle.

3.3 Discussion

The main findings from the results described in this chapter are:

- Ibrutinib and acalabrutinib inhibit or delay platelet aggregation in response to crosslinked IV.3 mAb and bacteria in a dose-dependent manner both in the presence and absence of plasma
- Ibrutinib and acalabrutinib inhibit platelet secretion of dense and α-granules in response to crosslinked IV.3 mAb and bacteria.

The results in this chapter expand on the knowledge of iBtk effects on platelet function, as previous studies mainly focused on pathways other than FcγRIIa. A previous investigation which explored the role of Btk and the effect of iBtks on the FcγRIIa pathway in platelets did not use bacteria.

In the first part of the present study, the effects of iBtks on CRP-induced platelet activation was evaluated, and ibrutinib was able to inhibit aggregation responses to high dose CRP in washed platelets This is in line with previous studies (Lee *et al.*, 2017) (Levade *et al.*, 2014) (Nicolson *et al.*, 2018a).

Ibrutinib is an irreversible Btk inhibitor (Shatzel *et al.*, 2017), as is acalabrutinib. However, studies have found the action of ibrutinib to be both irreversible and reversible in platelets. In other studies, experiments were performed in which platelets were first incubated with ibrutinib before being washed to remove the drug. The platelets were then stimulated with a GPVI agonists and showed an almost normal aggregation response, demonstrating the action of ibrutinib upon platelet function is not irreversible (Nicolson *et al.*, 2018a). However, further investigation of Btk phosphorylation found that such phosphorylation was irreversible, while the global tyrosine phosphorylation inhibition was reversible. Overall, the study concluded that ibrutinib and acalabrutinib have an irreversible action on Btk that delays platelet aggregation in response to GPVI agonists, but a reversible action on possible off-target proteins when employed at much higher concentrations of each drug which inhibits aggregation to GPVI agonists (Nicolson *et al.*, 2018a).

In the case of the FcγRIIa pathway, the inhibitory effects of iBtks could be due to (irreversible) inhibition of platelet Btk and/or (reversible) off-target inhibition of other kinases. Results presented in this chapter for inhibition of aggregation in PRP are obtained with a high concentration of acalabrutinib and ibrutinib which could induce off-target effects. However, iBtk concentrations needed to inhibit platelet aggregation in response to IV.3 XL and bacteria are lower than those needed to inhibit CRP induced platelet responses. This would suggest that the FcγRIIa pathway has a greater dependency on Btk than the GPVI pathway which has previously been observed (Goldmann *et al.*, 2019). In the study (Goldmann *et al.*, 2019), six iBtks were utilised to inhibit platelet activation in blood after FcγRIIa crosslinking by antibody as well as stimulation by HIT serum. The study found that maximal platelet responses, when stimulated with FcγRIIa agonists, were completely inhibited by iBtks, which is said to diverge from GPVI signalling in which an increase in GPVI agonist can overcome iBtk inhibition. The study only used low levels of iBtks which it claimed would be Btk-specific, as well as used fenebrutinib, which is meant to be highly specific for Btk.

Results in this chapter do not investigate the potential off-target effects of iBtks in either the GPVI or the FcγRIIa pathway. Possible proteins experiencing ibrutinib off-target effects include Src (Bye *et al.*, 2017) (Series *et al.*, 2019) (Nicolson *et al.*, 2018a), and Tec; the latter being known for its ability to substitute for Btk in the GPVI pathway (Atkinson, Ellmeier and Watson, 2003b).

αIIbβ3 has a role in the response of FcγRIIa to bacteria. When bacteria bind to FcγRIIa, the ITAM domain of the receptor is phosphorylated by Src kinases, which allows the binding and activation of Syk, resulting in the activation of a number of other proteins, including Btk, and the subsequent activation of PLCγ2. PLCγ2 activation results in the production of IP_3 and DAG which allows for the activation of PKC, all of which is essential for inside-out activation of αIIbβ3. Previous studies have shown that inhibition of αIIbβ3 prevents platelet activation to bacteria through FcγRIIa (Arman *et al.*, 2014). As such αIIbβ3 is expected to be playing a role in signalling when bacteria are present in the reactions in this chapter. While ibrutinib may be affecting Btk directly downstream of FcγRIIa signalling, it may also be inhibited downstream of αIIbβ3 and thus preventing outside-in signalling by the integrin required for the

platelet response to bacteria to take place. This inhibition of Btk downstream of αIIbβ3 preventing outside-in signalling has been previously reported in the GPVI pathway (Bye *et al.*, 2015a). However, though this could be an effect seen, IV.3 XL stimulated platelets, though still utilising the FcγRIIa receptor, do not require αIIbβ3 for aggregation to take place, yet these reactions are still inhibited and at a lower concentration of ibrutinib, suggesting that while αIIbβ3 inhibition may contribute to overall inhibition of platelet aggregation, it is not the only pathway to be inhibited downstream of FcγRIIa.

Due to many other studies available describing the effects of ibrutinib on platelets, each of which incubate platelets with iBtks for different amount of time varying from 5 minutes to 1 hour (Nicolson *et al.*, 2018a) (Levade *et al.*, 2014) (Series *et al.*, 2019) (Dobie *et al.*, 2019), this study strove to explore the effects of drug incubation time on platelet aggregation inhibition. Data in this chapter shows no difference between iBtk preincubation times and aggregation responses to both bacteria, and though there is a slight increase in lag time with both iBtks, this occurs at all drug concentration and time points with no significant difference between them.

These studies demonstrate that platelet aggregation and secretion occurring in response to FcγRIIa agonists are impaired by the iBtks ibrutinib and acalabrutinib in healthy donors. This data suggests that iBtks are possibly modulating platelet-mediated immune functions. Aggregation of platelets and other platelet-cell interactions are key in immunothrombosis, the process whereby the induction of thrombus formation allows the capture and killing of pathogens. One study shows fibrin clots used to capture *Streptococcus* and mediate bacterial killing, however, this study only used plasma and not platelets (Påhlman *et al.*, 2013), while another study shows the formation of microaggregates surrounding opsonised *E. coli* which is thought to aid in the facilitation of bacterial killing. Due to platelets being unable to undergo FcγRIIa-mediated aggregation responses to bacteria when inhibited by iBtks, such thrombi formation could occur over a longer duration of time. Instead, it could be possible that the platelets would rely on other immune cells in the presence of iBtks, such as monocytes which have been shown to secrete TF from microvesicles, to stimulate platelet aggregation to a pathogen to begin the process of

immunothrombosis, providing the other immune cells are also not affected by iBtk. Platelets contain substances in their granules which play a role in the capture and killing of pathogens, such as PF4, as well as in cross-talking with leukocytes including phagocytes. Thus, the inhibition of platelet granule release via iBtks could have a direct or indirect impact on bacterial burden in the blood, although this might be bacterial strain specific and requires further investigation.

In summary, this chapter demonstrates that the *in vitro* use of iBtks ibrutinib and acalabrutinib in healthy donors impairs platelet functions of aggregation and granule release in response to FcγRIIa agonists IV.3 XL and *S. aureus* Newman and *E. coli* RS218. This chapter raises the questions of the effects of ibrutinib on CLL patients treated with the drug, and what off target effects iBtks could potentially be exerting on the FcγRIIa pathway.

Chapter 4: The Effect of Ibrutinib on FcγRIIa Mediated Platelet-Bacteria Interactions in CLL

4.1 Introduction

Ibrutinib is an effective first-line treatment for CLL patients which is taken continuously until intolerance or disease progression. Consequently, circulating blood cells including platelets are constantly exposed to ibrutinib. The use of ibrutinib is associated with anti-haemostatic and anti-platelet effects as previously discussed in chapter 1.

Infections are common in CLL patients. The causes of the high risk of infection are multifactorial, including inherent humoral immunosuppression, age, and other co-morbidities. However, many clinical trials have reported an increased infection risk in CLL patients taking ibrutinib (refer to chapter 1 section 1.6.1).

As mentioned, age is a factor that could affect infection risk in CLL patients, with the median age of diagnosis being 72 years old in this study. There are many reasons aging contributes towards increased infections, such as chronic low grade inflammation, changes to immune receptors which come with age, changes in cell-mediated and humoral immunity, and degenerative changes associated with age (Gardner, 1980) (Haynes, 2020). However, it could also affect platelets as well. In healthy individuals, platelet count decreases with age (Segal and Moliterno, 2006). Platelets from the healthy elderly population are known to become hyperactive, and have a decreased bleeding time (Reilly and FitzGerald, 1986), a higher sensitivity to classical agonists (O'Donnell *et al.*, 2001) (Kasjanovová and Baláž, 1986) (Kasjanovova *et al.*, 1993), and increased plasma PF4 levels (Zahavi *et al.*, 1980).

Another factor contributing to infection risk in CLL patients is the levels and repertoire of endogenous IgGs, as IgG is required for FcγRIIa receptor activation. Hypogammaglobulinaemia is frequently seen in CLL patients. One such study states that 27.3%, 30.7% and 56.7% of patients had deficiencies in IgG, IgA and IgM respectively (Freeman *et al.*, 2013). An IgG subclass deficiency was seen in 64.6% of

patients, of which 28%, 19.3%, 52% and 22.7% were deficient in IgG1, IgG2, IgG3 and IgG4 respectively, while 27.3% of patients had hypogammaglobulinemia (Freeman *et al.*, 2013). Another study found 26% of patients suffered from hypogammaglobulinemia (Parikh *et al.*, 2015), while another found 58% of patients with abnormal IgM, IgG and IgA levels (Ishdorj *et al.*, 2019). Overall, studies tend to give variable ranges, with decreases for IgA between 12-68%, IgM 4-56% and IgG 9-76% (Lee *et al.*, 1987) (Shvidel *et al.*, 2014) (Hansen *et al.*, 1994) (Andersen *et al.*, 2016) (Crassini *et al.*, 2018). Therapy in the treatment of relapsed CLL can also cause an increased infection risk compared to if the same therapy was used as a front-line treatment (Teh *et al.*, 2018) (Thursky *et al.*, 2006).

In CLL, platelet count is used in the determination of CLL clinical stage, with low platelet counts being included in both the severe stages in the Rai and Binet staging systems (Rai *et al.*, 1975) (Binet, Leporrier, *et al.*, 1977). While this is suggestive that CLL progression effects platelets, not much is known about the platelets of CLL patients except in the cases of ibrutinib, and more recently other iBtk, treated CLL for which there have been many studies looking into the effects of ibrutinib downstream of αIIbβ3, GPVI and GPIb as a cause of increased bleeding risk in these patients (Bye *et al.*, 2015a) (Kamel *et al.*, 2015) (Levade *et al.*, 2014). Despite these studies into the effects of iBtks on platelet function in haemostasis and thrombosis, the immune functions of CLL platelets have not been studied.

FcγRIIa is an important immune receptor in platelets and is one of the elements by which platelets can interact with bacteria. Upon time of writing, there are no studies in which FcγRIIa function in CLL platelets has been tested. In chapter 3 I showed iBtks inhibit FcγRIIa responses in platelets of healthy donors *in vitro.* Considering these results and the association between ibrutinib treatment of CLL patients and infection risk, in this chapter I consider the effects on *in vivo* exposure of ibrutinib on FcγRIIa-mediated platelet function.

Hypothesis

Platelet immune functions in CLL are unknown. In contrast to the *in vitro* results obtained with platelets from healthy donors in chapter 3, there are other factors to

consider for studying CLL patients which could alter platelet responses to bacteria, such as age, endogenous IgG levels and repertoire, and other comorbidities. Another factor to consider in an elderly CLL patient cohort is the effect of concurrent medications. Due to patient age, it is expected for patients to have multi-morbidity. Due to this, a range of drugs may possibly be taken in addition to ibrutinib. Many drug classes are known to have off-target effects on platelet function, which may affect the platelet responses to bacteria.

The hypothesis to be investigated in this chapter is: CLL platelets have an impaired immune response to bacteria that is worsened by ibrutinib treatment.

Aims of this chapter

The key questions aimed to be answered within this chapter are:

- Do platelets from CLL patients respond to bacteria?
- Does ibrutinib treatment impair platelet responses to bacteria in CLL patients?
- Does ibrutinib treatment impair FcγRIIa-mediated platelet activation in CLL patients?

To answer these questions, LTA and PF4 ELISAs were carried out for CLL platelets derived from both ibrutinib-treated and ibrutinib-untreated patients in the presence of plasma to account for IgG levels and repertoires specific for the disease. Crosslinked IV.3 mAb was used to directly ligate the FcγRIIa receptor, whilst *S. aureus* Newman and *E. coli* RS218 were bacteria chosen as potential stimulants as both species cause FcγRIIa-dependent platelet activation (Cox, Kerrigan and Watson, 2011) (Watson *et al.*, 2016). When platelet activation was seen, further inhibitory conditions were used as determined in chapter 3. Other non-FcγRIIa activating agonists were used for both LTA and in ELISAs: TRAP6 amide, ADP and CRP, which all have been previously studied in CLL platelet responses (Kamel *et al.*, 2015) (Bye *et al.*, 2017).

To investigate platelet response of bacteria scavenging, scavenging assays were performed in which platelets derived from healthy controls, ibrutinib-treated and ibrutinib-untreated CLL patients were spread on bacteria-coated coverslips before imaging via confocal fluorescence microscopy and the scavenging analysed.

To explore the possible effects of ibrutinib-treatment on surface receptor levels in platelets which could affect platelet responses to bacteria, flow cytometry to detect FcγRIIa, αIIbβ3 and GPVI was carried out on PRP samples obtained from ibrutinib-treated and ibrutinib-untreated CLL patients and compared to healthy donor-derived PRP.

4.2 Results

4.2.1 Patient cohort characteristics

34 ibrutinib-untreated and 32 ibrutinib-treated CLL patients were used throughout the studies, with their characteristics summarised in Table 4.1. Healthy controls were opportunistic and as such were not age or sex matched to either CLL cohort, furthermore the average age of the healthy controls will be significantly younger than either CLL groups. Differences are seen within the types of drugs taken by each group of patients in Table 4.2. 61% of ibrutinib-treated CLL patients were taking antibiotics which are mainly prescribed as a prophylactic. As can be seen in Table 4.3, the number of concurrent medications for comorbidities for both ibrutinib-untreated and ibrutinib-treated CLL patients was similar, with the median number being 3 and 4 respectively. The platelet concentrations in PRP were similar to healthy controls for both ibrutinib-treated and ibrutinib-untreated CLL patients (Figure 4.1).

Table 4.1. Characteristics of CLL patients used throughout this study.

	Overall (n=63)		Ibrutinib-untreated CLL (n=32)		Ibrutinib-treated CLL (n=31)		P value
Sex:							
Male	41	(65%)	20	(62%)	21	(68%)	0.79
Female	22	(35%)	12	(38%)	10	(32%)	
Age (years)	71	(45-86)	71	(45-86)	71	(55-82)	0.78
17p deletion							
Positive	5	(8%)	0	(0%)	5	(16%)	0.02
Negative	58	(92%)	32	(100%)	26	(84%)	
Trisomy 12							
Positive	0	(0%)	0	(0%)	0	(0%)	>0.99
Negative	63	(100%)	32	(100%)	31	(100%)	
11q deletion							
Positive	7	(11%)	1	(6%)	6	(19%)	0.05*
Negative	56	(89%)	31	(94%)	25	(81%)	
13q deletion							
Positive	13	(21%)	0	(0%)	13	(42%)	<0.001*
Negative	50	(79%)	32	(100%)	18	(58%)	
β2 microglobulin	2.74	(1.4-6.5)	2.74	(1.4-6.5)	2.76	(1.5-6.3)	0.4
IgG at Diagnosis (g/L)	9.25	(3.1-22)	9.23	(3.1-21.5)	9.26	(5.2-22)	0.9
CD38							
Positive	20	(32%)	9	(28%)	11	(35%)	0.6
Negative	43	(68%)	23	(72%)	20	(65%)	
Binet stage#							
A	46	(73%)	21	(66%)	26	(81%)	0.11
B	4	(6%)	4	(13%)	0	(0%)	
C	11	(17%)	6	(19%)	5	(16%)	
Time from Diagnosis (years)	6.2	(0.1-23.4)	3.6	(0.1-13.9)	8.9	(1-23.4)	<0.001*
Time on Ibrutinib (years)	-		-		1.5	(0.1-3.4)	-
Number of Medications excluding ibrutinib	4	(0-9)	3	(0-9)	4	(1-9)	0.08
Haemoglobin (g/L)	129.7	(87-163)	125.8	(87-152)	133.9	(95-163)	0.08
White Blood Cell Count (x10^9/L)	58.16	(2.7-379)	68.95	(4.1-379)	46.64	(2.7-319)	0.008*
Lymphocyte (x10^9/L)##	51.19	(0.6-372)	93.91	(16-372)	24.9	(0.6-279)	<0.001*
Platelet Count (x10^9/L)	164.4	(39-378)	178.7	(39-378)	149.1	(75-253)	0.06*

All values are at time of sample collection except for IgG and β2 microglobulin levels, cytogenetic tests, and CD38 positivity which is at time of diagnosis. Local reference ranges for normal adult whole blood parameters were obtained from Hull University Teaching Hospitals NHS Trust (UK): Hb, 120-160 g/L for women and 135-175 g/L for men; WCC, 4-11x10^9/L; lymphocyte count, 0.8-3.4x10^9/L; platelet count, 150-400x10^9/L; β2 microglobulin, 1.2-2.4 mg/L; IgG serum concentration, 6-16 g/L.
For continuous variables, P values were obtained by Mann-Whitney test for non-parametric or student t-test for parametric data. For non-continuous variables, P values were obtained by chi-square test or Fishers exact test. P≤0.05 was significant. Graph shows median (range).
[#]Binet Stage ibrutinib-untreated patients n=33, ibrutinib-treated patients n=31.
[##]Lymphocyte counts ibrutinib-untreated patients n=13, ibrutinib-treated patients n=16. *Significant difference (p ≤0.05).

Table 4.2. Concurrent medication types in CLL patients enrolled in the study.

Drug Type	Ibrutinib-untreated patients (n=32)		Ibrutinib-treated patients (n=31)	
Antidepressant	6 (18%)	Sertraline (2), Amitriptyline (2), Fluoxetine (1), Paroxetine (1)	5 (16%)	Sertraline (1), Amitriptyline (4), Fluoxetine (1)
Antidiarrheal Agent	1 (3%)	Loperamide (1)	0 (0%)	-
Hyperlipidaemia Statin	13 (39%)	Atorvastatin (7), Simvastatin (6)	8 (26%)	Atorvastatin (5), Rosuvastatin (1), Simvastatin (1), Pravastatin (1)
Hypertension	15 (45%)	Amlodipine (6), Ramipril (4), Perindopril (1), Bisoprolol (2), Bendroflumethiazide (4), Lisinopril (2), Enalapril (2), Verapamil (1), Angitil (1), Carvedilol (1), Atenolol (1), Losartan (3), Indapamide (1)	17 (55%)	Amlodipine (7), Ramipril (3), Perindopril (1), Bisoprolol (3), Lisinopril (2), Losartan (4), Entresto (1), Adipine (1), Carvedilol (1), Lercanidipine (1), Indapamide (1)
Erectile Dysfunction	2 (6%)	Sildenafil (2)	1 (3%)	Sildenafil (1)
Gastric and Duodenal Ulceration	7 (21%)	Omeprazole (3), Lansoprazole (4)	13 (42%)	Omeprazole (3), Lansoprazole (8), Ranitidine (3)
Arthritis	0 (0%)	-	1 (3%)	Etoricoxib (1)
Osteoporosis	1 (3%)	Alendronate (1)	1 (3%)	Alendronate (1)
Anti-inflammatory	2 (6%)	Budesonide (1), Naproxen (1)	0(0%)	-
Vitamin and Iron Deficiency	5 (15%)	Calceos (2), Ferrous fumarate (1), Accrete D3 (1), Thiamine (1)	5 (16%)	Folic acid (2), Ferrous fumarate (2), Evacal D3 (1)
Bladder and Urinary Disorders	5 (18%)	Tamsulosin (4), Doxazosin (1),	7 (23%)	Tamsulosin (1), Mirabegron (1), Solifenacin (1), Tolterodine (2), Finasteride (1), Alfuzosin (1)
Hormone/Hormone Altering	2 (6%)	Oestradiol (1), Finasteride (1)	4 (13%)	Levothyroxine (3), Dutasteride (1)
Analgesics	6 (18%)	Co-codamol (4), Paracetamol (2)	14 (45%)	Co-codamol (2), Tramadol (2), Oxycodone (2), Codeine (3), Paracetamol (6), Ibuprofen (1)

Antibiotic	0 (0%)	-	19 (61%)	Amoxicillin (1), Co-trimoxazole (17), Methenamine hippuarate (1)
Antihistamine	0 (0%)	-	2 (6%)	Fexofenadine (1), Hydroxyzin (1)
Sedative	1 (3%)	Zopiclone (1)	0 (0%)	-
Chemotherapy	0 (0%)	-	4 (13%)	Venetoclax (4)
Hyperuricaemia drugs	1 (3%)	Allupurinol (1)	2 (6%)	Allupurinol (2)
Anti-Vertigo/Motion Sickness	1 (3%)	Hyoscine (1)	1 (3%)	Betahistine (1)
Anti-Diabetic	6 (18%)	Sitagliptin (1), Metformin (5), Gliclazide (1), Dapagliflozin (1), irbesartan (1)	1 (3%)	Sitagliptin (1)
Anti-Malarial (muscle cramp treatment)	1 (3%)	Quinine (1)	2 (6%)	Quinine (2)
Antithrombotic	5 (15%)	Apixaban (5)	0 (0%)	-
Epilepsy	2 (6%)	Sodium valproate (1), Clonazepam (1), Gabapentin (1)	0 (0%)	-
Anti-Lipemic	1 (3%)	Bezafibrate (1)	0 (0%)	-
Diuretic	4 (12%)	Furosemide (2), Bumetanide (1), Spironolactone (1)	1 (3%)	Spironolactone (1)
Gout	0 (0%)	-	1 (3%)	Allopurinol (1)
Laxative	0 (0%)	-	1 (3%)	Laxido (1)
Mucolytic	0 (0%)	-	1 (3%)	Carbocysteine (1)
Muscle Relaxant	1 (3%)	Methocarbamol (1)	0 (0%)	-
Anti-asthmatic	1 (3%)	Montelukast (1)	0 (0%)	-

Concurrent medications CLL patients were treated with during the study were categorised by drug type based on the British National Formulary (Joint Formulary Committee. (2018). BNF 76th Ed. Pharmaceutical Press). Table depicts number of patients taking medications from that drug type (n) and percentage (%), as well as specific medications taken in each drug category. Some patients take more than one medication per drug type.

Table 4.3. Number of drugs, excluding ibrutinib, in CLL patients enrolled in the study.

Number of Drugs	Ibrutinib-untreated patients (n=32)		Ibrutinib-treated patients (n=31)	
	No. of Patients	Percentage	No. of Patients	Percentage
0	7	21.87	0	0
1	4	12.50	6	19.35
2	5	15.63	5	16.13
3	5	15.63	1	3.23
4	2	6.25	4	12.90
5	2	6.25	7	22.58
6	3	9.38	3	9.68
7	2	6.25	2	6.45
8	1	3.13	2	6.45
9	1	3.13	1	3.23

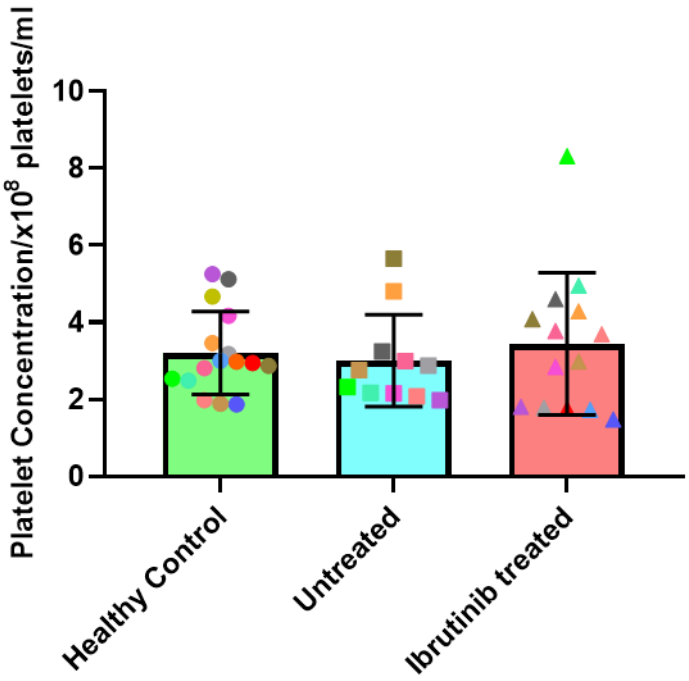


Figure 4.1 Platelet sample preparations from CLL groups and healthy controls have similar platelet concentrations. Platelets concentrations from healthy controls, ibrutinib-untreated and ibrutinib-treated CLL PRP preparations were determined in platelets/ml by a Z1 Coulter particle counter, and no significant difference was found between the three groups. Data shows mean ±SD.

4.2.2 Ibrutinib-treated CLL patients have impaired platelet aggregation responses to bacteria and antibody-mediated FcγRIIa crosslinking

To ascertain if ibrutinib-untreated CLL patient platelets aggregate in response to bacteria in a similar manner to that observed in healthy controls, LTA was performed. The experiments were also carried out for ibrutinib-treated CLL platelets to see if platelet aggregation responses were diminished due to the *in vivo* effects of ibrutinib.

PRP was isolated from whole blood for all donors, and no significant difference was found between platelet concentrations of healthy controls, ibrutinib-untreated and ibrutinib-treated CLL (Figure 4.1). PRP was stimulated with either IV.3 XL, *S. aureus* Newman or *E. coli* RS218. IV.3 XL platelet aggregation was significantly decreased for ibrutinib-treated CLL patients in comparison to both healthy controls and ibrutinib-untreated CLL patients ($P<0.001$ in both instances) (Figure 4.2). This demonstrates inhibition of ibrutinib on FcγRIIa crosslinking with antibody.

This significant reduction in platelet aggregation in ibrutinib-treated CLL patients was also true for *E. coli* RS218 stimulation ($P<0.001$ for ibrutinib-treated CLL versus healthy controls, and $P=0.07$ for ibrutinib-treated CLL versus ibrutinib-untreated CLL).

While platelet aggregation resulting from *S. aureus* Newman stimulation saw a significant decrease for ibrutinib-treated CLL platelets ($P<0.001$ for ibrutinib-treated CLL versus healthy controls, and $P=0.006$ for ibrutinib-treated CLL versus ibrutinib-untreated CLL), there was also a significant decrease with ibrutinib-untreated CLL patients compared to healthy controls ($P=0.006$). In the absence of the ibrutinib inhibitory effect in untreated patients, such reduced responses may be due to concurrent hypogammaglobulinaemia limiting the amount or type of IgGs available to support platelet aggregation via FcγRIIa in response to *S. aureus* Newman. This would explain why a similar trend is not seen in the responses of ibrutinib-untreated CLL patients with IV.3 XL, which does not require human IgGs to cause activation.

Overall, this confirms that ibrutinib-untreated CLL patients can respond to bacteria in terms of aggregation, while ibrutinib-treated CLL patients see a significant reduction in aggregation.

4.2.3 CLL patients have similar aggregation responses to major platelet receptor agonists to those observed in healthy controls

As evidenced in Table 4.3, the majority of CLL patients participating in this study were taking multiple medication for various comorbidities, which is not a characteristic of healthy controls, and were typically older than the latter, meaning that these two groups might not be comparable in terms of platelet function. To investigate this, a comparison of platelet aggregation responses in healthy controls, ibrutinib-untreated, and ibrutinib-treated CLL patients was performed. Agonists to major platelet receptors were employed in this analysis including: TRAP6 amide to the PAR1 receptor, CRP to the GPVI receptor, and ADP to P2Y1 and P2Y12 receptors. This analysis would give an overview as to the role of patient specific factors in platelet aggregatory responses.

PRP was isolated from whole blood from all donors and stimulated with the specified agonist. All agonists showed similar aggregation levels with any differences being non-significant (Figure 4.1). This suggests that patient specific factors are having minimal to no effects on platelet aggregation. Additionally, results show CRP stimulation of ibrutinib-treated CLL platelets is significantly decreased in comparison to both healthy controls and ibrutinib-untreated CLL patients ($P<0.001$ in both cases), which is in line with previous studies (Kamel *et al.*, 2015) (Nicolson *et al.*, 2018a) (Bye *et al.*, 2015a) (Bye *et al.*, 2017).

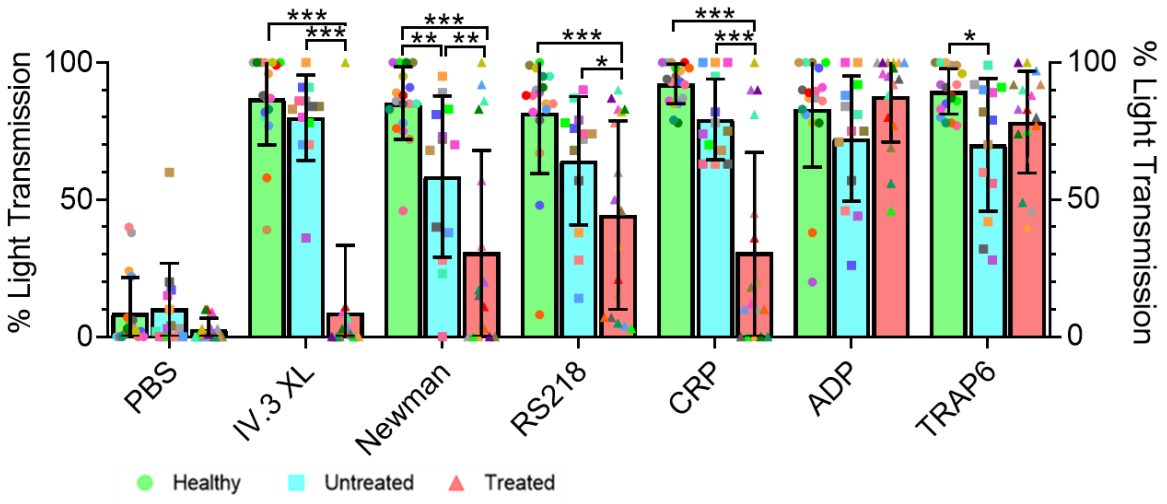


Figure 4.2. CLL ibrutinib-treated patients have impaired aggregatory responses to bacteria and IV.3 mAb-mediated cross-linking of FcγRIIa. PRP from either healthy controls, ibrutinib-treated or ibrutinib-untreated CLL patients was stimulated with either PBS, 10µM ADP, 3µM TRAP6 amide or 3µg/ml CRP and recorded for 10 minutes, or 4µg/ml mAb IV.3 for 2 minutes followed by 30µg/ml $F(ab')_2$ rabbit anti-mouse IgG, *S. aureus* Newman, *E. coli* RS218 and recorded for 20 minutes. Data shown is maximum % light transmission. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001.

4.2.4 Ibrutinib-untreated CLL patients have a decreased lag time to aggregation in response to bacteria

Although platelets derived from ibrutinib-untreated CLL aggregated in response to bacteria, it was still possible that the lag time that it takes platelets to aggregate could be affected by patient specific factors such as medication, age and possibly plasma IgG content. The lag times from aggregation of healthy controls and ibrutinib-untreated CLL patients to *S. aureus* Newman and *E. coli* RS218 were compared in Figure 4.3. In response to stimulation with both bacteria, lag times of ibrutinib-untreated CLL patients were significantly shorter than that of healthy control platelets, with P=0.029 for *S. aureus* Newman, and P=0.0381 for *E. coli* RS218. This suggests that the platelets in CLL can respond quicker to bacteria in terms of a lag time which is quicker by seconds while levels of aggregation remain the same as that of healthy controls. Though this may not have a major physiological effect, it could suggest there may be molecular mechanism changes between the healthy controls and CLL platelets which are not investigated in this study.

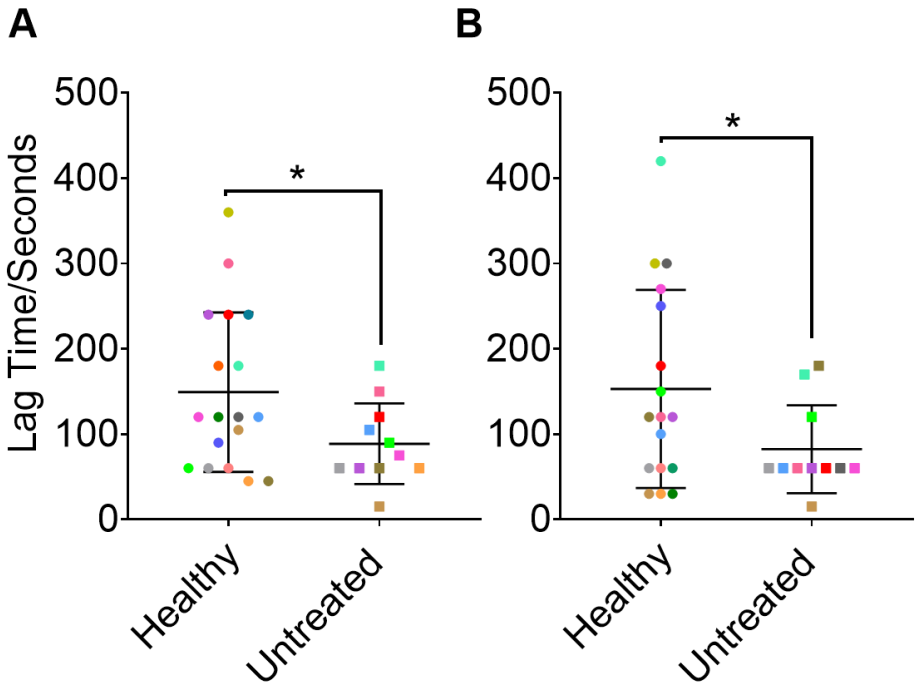


Figure 4.3. Ibrutinib-untreated CLL patients have reduced lag times until platelet aggregation in response to bacteria compared to healthy controls. PRP from either healthy controls or ibrutinib-untreated CLL patients was stimulated with either (A) *S. aureus* Newman or (B) *E. coli* RS218 and time taken from point of injection to start of aggregation was measured. Data shows mean ±SD. *P<0.05.

4.2.5 IgG addition can rescue platelet aggregation in response to *E. coli* RS218 in ibrutinib-untreated CLL patients

CLL is commonly associated with hypoagammaglobulinaemia. The FcγRIIa pathway requires IgG for platelet activation and aggregation to occur in response to *S. aureus* Newman (Arman *et al.*, 2014) and *E. coli* RS218 (Watson *et al.*, 2016) stimulation. Whilst most ibrutinib-untreated CLL patients aggregate normally to bacteria (Figure 4.2) possibly suggesting that IgG levels or repertoires are adequate to sustain activation responses, one patient showed no aggregation in response to *E. coli* RS218. To investigate if this was secondary to low IgG levels or repertoire, PRP was incubated with either vehicle, hIgGs or hIgGs and eptifibatide (αIIbβ3 inhibitor) for 2 minutes before stimulation by *E. coli* RS218. hIgGs used in assays were commercially available IgGs purified and pooled from healthy donors. As seen in Figure 4.4, the addition of hIgGs supported platelet aggregation in response was *E. coli* RS218. This suggests that for this individual, and possibly other ibrutinib-untreated CLL patients, plasma IgG composition might compromise aggregation through the FcγRIIa pathway. However, IgG levels at sample collection for this patient are unknown, but at diagnosis 4 years prior was 6.2g/L which is within normal IgG serum concentrations.

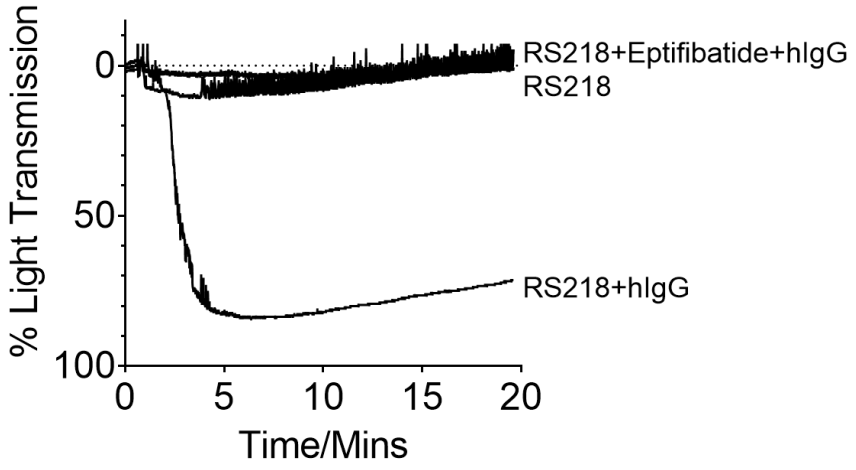


Figure 4.4. Addition of hIgG can rescue platelet aggregation to E. coli RS218 in a case of ibrutinib-untreated CLL. PRP from an ibrutinib-untreated CLL patient who had previously failed to aggregate in response to *E. coli* RS218 was either incubated with 0.2mg/ml hIgGs purified and pooled from healthy donors, 0.2mg/ml hIgGs plus 9µM eptifibatide, or PBS for 2 minutes, before stimulation with *E. coli* RS218 for 20 minutes and measured by LTA. n=1.

4.2.6 Ibrutinib-untreated CLL platelet aggregation responses to FcγRIIa agonists are inhibited by FcγRIIa pathway inhibitors

Though FcγRIIa is a well characterised receptor by which bacteria initiate platelet activation, other pathways are also known. Little is known about how CLL patient platelets react to bacteria, and as such it was important to investigate whether the FcγRIIa pathway is still functional in these patients.

To assess the FcγRIIa pathway functionality, ibrutinib-untreated CLL patient derived PRP was incubated with the following inhibitors of the FcγRIIa pathway: IV.3 mAb to inhibit the FcγRIIa receptor, dasatinib to inhibit Src kinases, and ibrutinib to inhibit Btk. PRP was then stimulated with either IV.3 XL (in these reactions inhibition by IV.3 mAb is not possible), *S. aureus* Newman, or *E. coli* RS218.

Figure 4.5 demonstrates that bacteria-induced aggregation was significantly inhibited by all inhibitors tested, with P values for *S. aureus* Newman with IV.3 mAb, dasatinib and ibrutinib being <0.001 in all cases, and P values for *E. coli* RS218 with IV.3 mAb, dasatinib and ibrutinib being 0.004, <0.001 and 0.01 respectively. This indicates that platelet aggregation is FcγRIIa mediated in response to bacteria. Figure 4.5 shows that, in CLL samples, ibrutinib affects CLL responses to bacteria *in vitro* as seen for healthy donors in chapter 3. Dasatanib also significantly blocked IV.3 XL in CLL (P<0.001), indicating that the FcγRIIa pathway is still functional up until at least Src kinases recruitment.

Stimulation by TRAP6 amide was used as a negative control as the thrombin receptor PAR1 is related to G-protein signalling rather than tyrosine kinase signalling. The inhibitors tested had no effect on TRAP6 amide induced platelet aggregation, except for dasatinib. Dasatanib has previously been found to significantly inhibit platelet aggregation to low concentrations of thrombin, and weakly affect platelet aggregation in response to high concentrations of thrombin (Gratacap *et al.*, 2009).

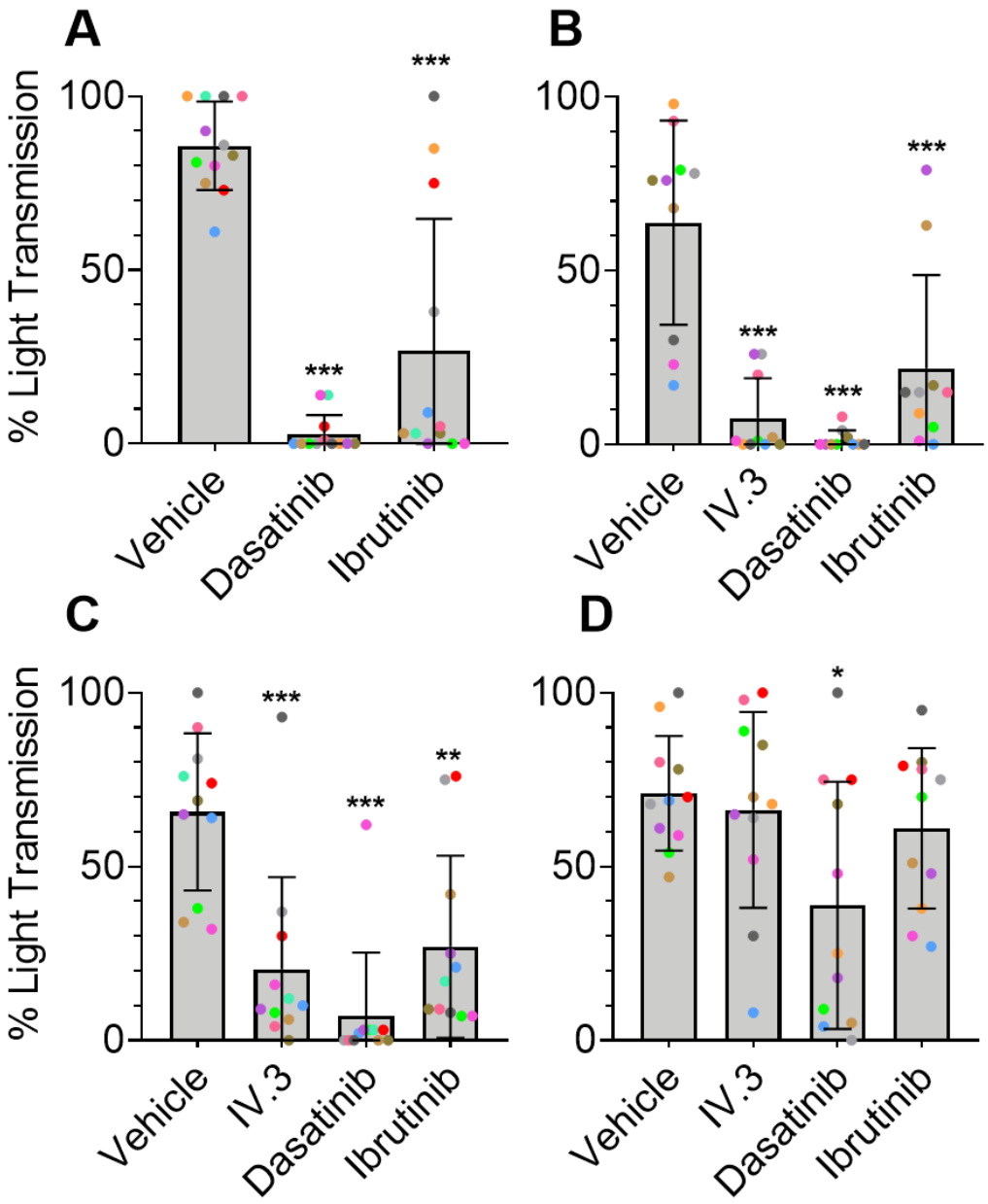


Figure 4.5. Ibrutinib-untreated CLL platelet aggregation in response to bacteria is FcγRIIa dependent. PRP from ibrutinib-untreated CLL patients was incubated with either vehicle or 5μM ibrutinib, 20μg/ml mAb IV.3, or 4μM dasatinib, before stimulation with either (A) 4μg/ml mAb IV.3 followed by 30μg/ml $F(ab')_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218 for recorded and 20 minutes, or (D) 3μM TRAP6 amide recorded for 10 minutes. Graphs show final aggregation. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 compared to vehicle.

4.2.7 Platelet aggregation responses to bacteria observed in some ibrutinib-treated CLL patients are inhibited by FcγRIIa pathway inhibitors

Results presented in Figure 4.2 show that platelets from 4 ibrutinib-treated CLL patients are still able to aggregate in response to bacteria and IV.3 XL. In these instances, PRP was incubated with the inhibitors IV.3 mAb, dasatinib, and ibrutinib, before activation with FcγRIIa agonists (Figure 4.6). This was performed to ascertain if an alternate pathway to FcγRIIa was being utilised, or if ibrutinib was not having the effect seen normally.

For all 4 donors for which this experiment was performed (Figure 4.6) *E. coli* RS218 was the consistent agonist used due to each of the patients still displaying aggregation in response to this bacteria. Moreover, 2 donors were tested for *S. aureus* Newman, and 1 donor for IV.3 XL, as in these cases platelet from the patients also aggregated in response to these agonists. TRAP6 amide was used as a control in all cases. For donor 1 both dasatinib and ibrutinib could inhibit aggregation to IV.3 XL. For donor 1 and 2 stimulated with *S. aureus* Newman, all FcγRIIa inhibitors inhibited aggregation. In all donors stimulated with *E. coli* RS218, IV.3 mAb and dasatinib inhibited aggregation, though in donors 1, 3 and 4 ibrutinib failed to do this. This data suggests the possibility that platelets can respond to *E. coli* RS218 in an FcγRIIa-dependent way which can also be Btk independent.

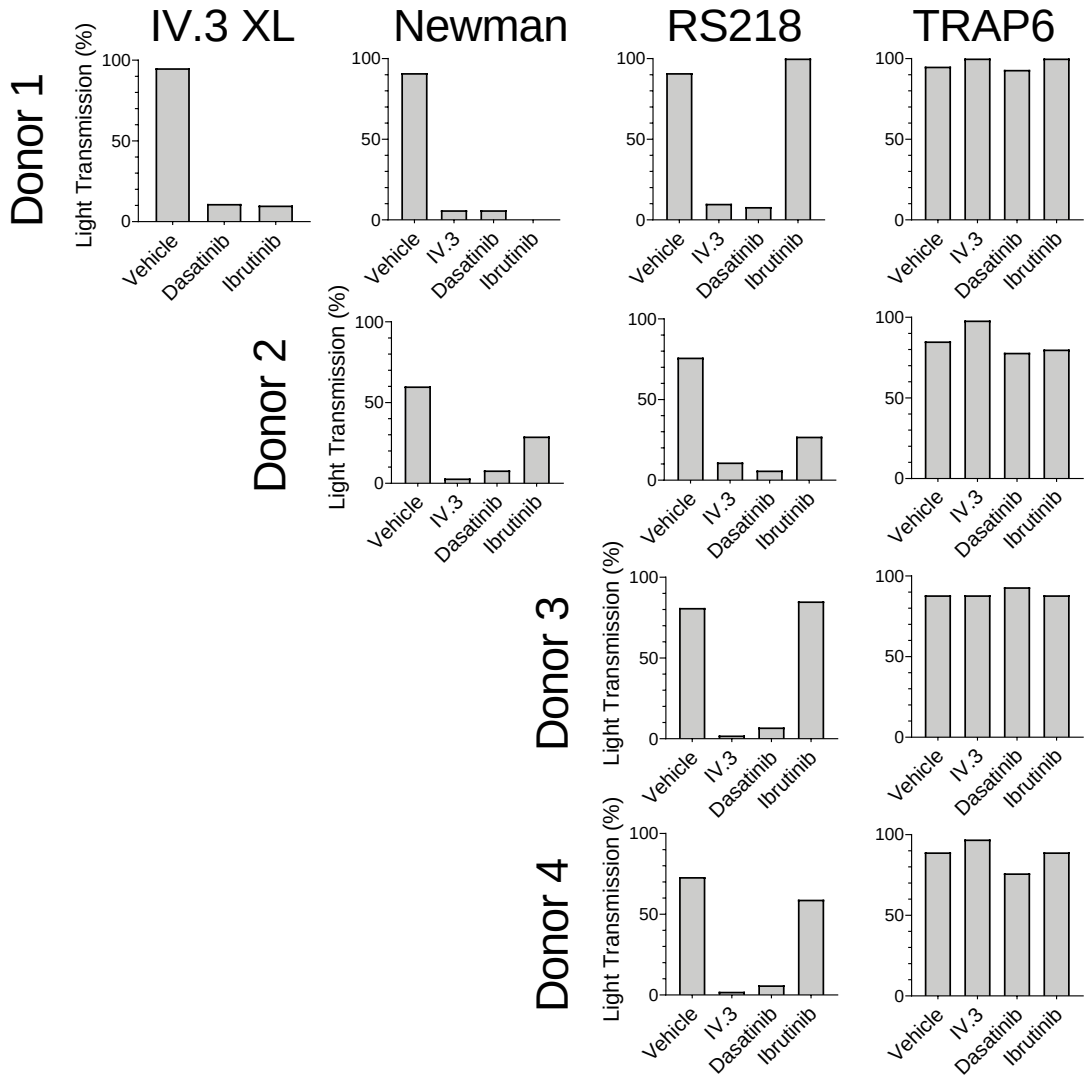


Figure 4.6. Bacteria-induced aggregation of platelets derived from ibrutinib-treated CLL patients is FcγRIIa-dependant. In ibrutinib-treated CLL samples where platelet aggregation to bacteria was observed (see Figure 4.2), PRP was incubated with either vehicle or 5μM ibrutinib, 20μg/ml mAb IV.3 or 4μM dasatinib, before stimulation with either 4μg/ml mAb IV.3 followed by 30μg/ml $F(ab')_2$ rabbit anti-mouse IgG, *S. aureus* Newman, *E. coli* RS218 recorded for 20 minutes, or 3μM TRAP6 amide recorded for 10 minutes. Results from individual donors are shown.

4.2.8 Ibrutinib-treated CLL patients have impaired PF4 release in response to FcγRIIa receptor agonists

Release of platelet granules is important for a variety of platelet functions, especially in infections and immunity due to the release of bactericidal substances as well as the release of signalling molecules, like chemokines, to other immune cells. As such, it is important to study CLL platelet secretion in response to bacteria and how ibrutinib may affect this. As changes in aggregation to FcγRIIa agonists were seen in ibrutinib-treated patients (Figure 4.2) those reactions were stopped and an ELISA for PF4 was performed on the platelet-free supernatant from these reactions. This was done to see if ibrutinib was inhibiting granule release in platelets in response to FcγRIIa agonist.

PF4 release in response to IV.3 XL displayed a significant decrease in ibrutinib-treated CLL patients compared to ibrutinib-untreated CLL patients ($P<0.001$) (Figure 4.7). Although an inhibitory trend was observed in PF4 release in response to *E. coli* RS218 and *S. aureus* Newman between ibrutinib-untreated CLL patient and ibrutinib-treated CLL patients, this was not statistically significant ($P=0.136$ and 0.206 respectively).

The CLL patient cohort in this study was known to be taking a variety of medications and be of an older age. Some medications (other than ibrutinib) may inhibit platelet granule release (Prowse, Pepper and Dawes, 1982), and, therefore, PF4 release by CLL platelets was also compared to healthy control platelets and found to be comparable (Figure 4.7 and chapter 3 Figure 3.14).

4.2.9 PF4 release in response to major platelet agonists is affected by in ibrutinib therapy in CLL platelets

To investigate the effect of ibrutinib therapy on platelet PF4 release induced by FcγRIIa receptor-independent agonists, platelet aggregation reactions seen in Figure 4.2 were stopped by the addition of prostacyclin and centrifuged to gain a platelet free supernatant. A PF4 ELISA was performed on this supernatant. A significant difference is shown in Figure 4.7 between ibrutinib-untreated CLL patients and ibrutinib-treated patients in terms of PF4 release in response to CRP (P=0.02) but not to ADP and TRAP6 amide. Healthy controls are included for PBS and IV.3 XL sample comparison, and the general levels of PF4 release for ibrutinib-untreated CLL platelets are similar to that seen for healthy donors in Figure 3.14.

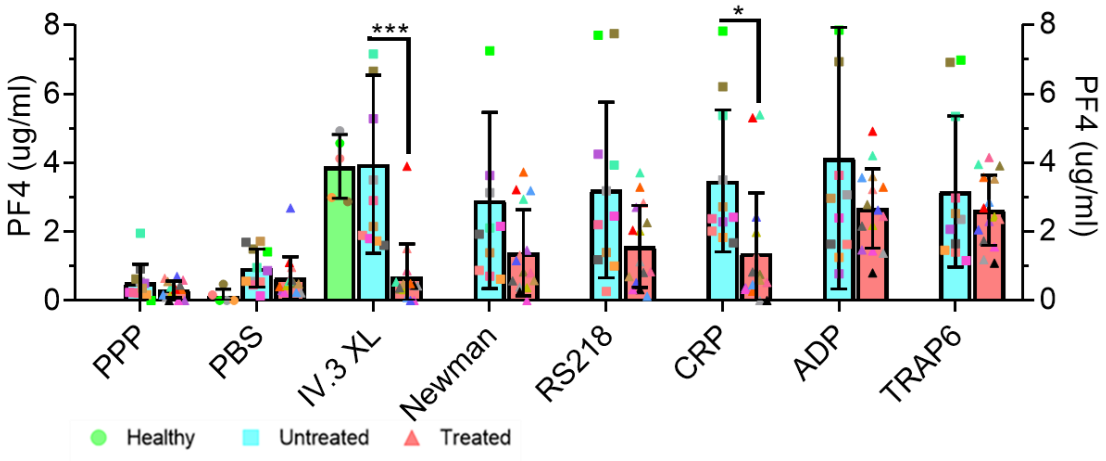


Figure 4.7. Platelets from ibrutinib-treated CLL patients have an impaired PF4 release response to bacteria and direct cross-linking of the FcγRIIa receptor by antibodies. Levels of PF4 were measured by ELISA in supernatants collected from PRP from either healthy controls, ibrutinib-untreated, or ibrutinib-treated CLL patients after stimulation with either PBS, 10µM ADP, 3µM TRAP6 amide, 3µg/ml CRP, 4µg/ml mAb IV.3 followed by 30µg/ml F(ab')$_2$ rabbit anti-mouse IgG, *S. aureus* Newman or *E. coli* RS218. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001.

4.2.10 Ibrutinib-untreated CLL platelet PF4 release in response to bacteria can be inhibited by FcγRIIa pathway inhibitors

As previously mentioned in 4.2.6, the FcγRIIa receptor pathway is not the only pathway through which bacteria can initiate platelet activation.

To investigate if the FcγRIIa pathway remains functional in CLL patients in terms of α-granule release, PRP derived from ibrutinib-untreated CLL patients was incubated with FcγRIIa pathway inhibitors: IV.3 mAb to inhibit the FcγRIIa receptor (not used in IV.3 XL reactions), dasatinib to inhibit Src kinases, and ibrutinib to inhibit Btk. PRP was then stimulated with either IV.3 XL, *S. aureus* Newman, or *E. coli* RS218. A platelet-free supernatant was then collected and an ELISA for PF4 was performed.

As seen in Figure 4.8, in the TRAP6 amide controls, no significant inhibition of PF4 release was seen. In contrast, all reactions to the FcγRIIa agonists were significantly inhibited by the FcγRIIa inhibitors. This suggests that the FcγRIIa pathway is utilised in CLL patients to mediate α-granule secretion.

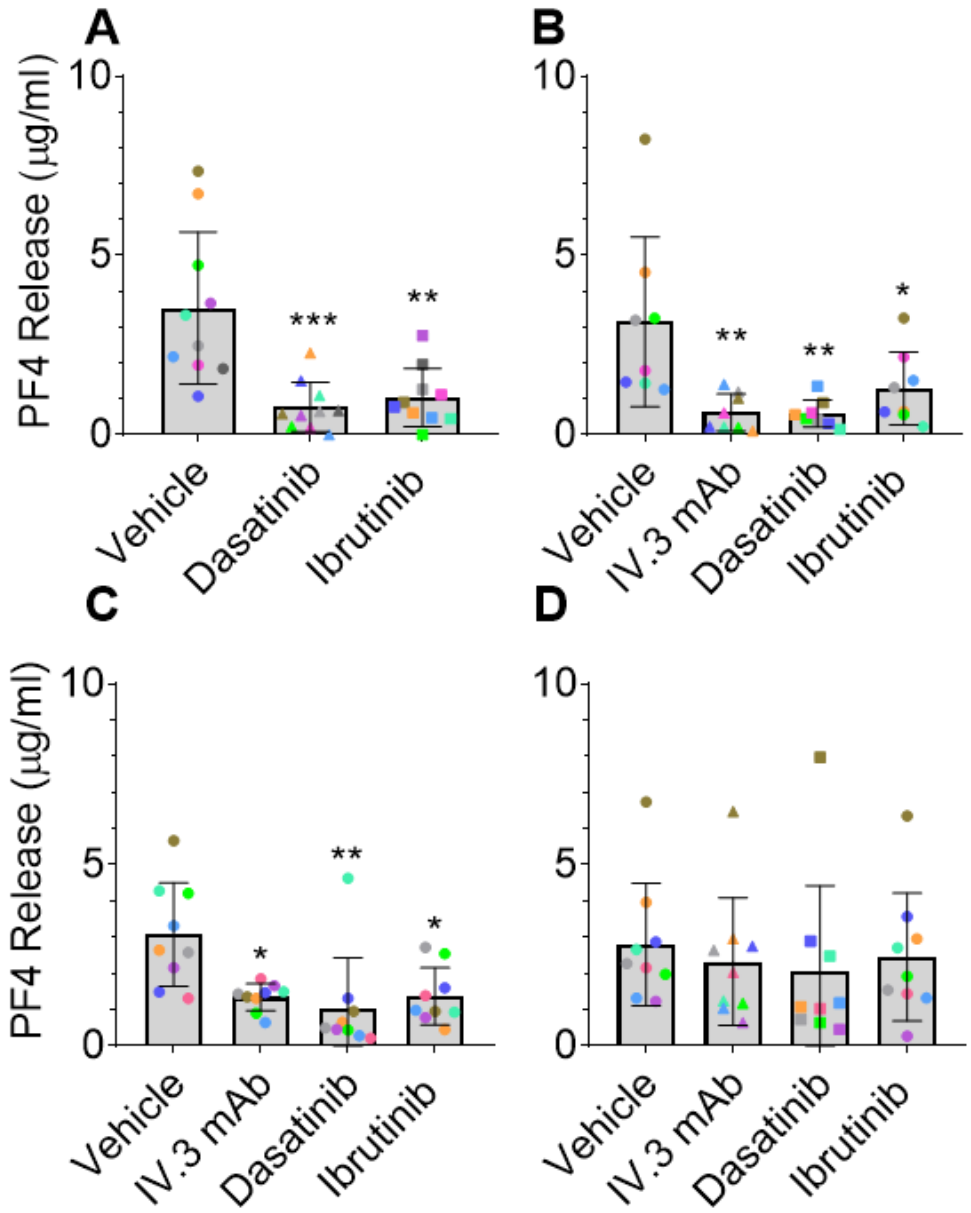


Figure 4.8. Ibrutinib-untreated CLL platelet PF4 release in response to bacteria is FcγRIIa dependent. PRP from ibrutinib-untreated CLL patients was incubated with either vehicle, 20µg/ml mAb IV.3, or 4µM dasatinib or 5µM ibrutinib before stimulation with either (A) 4µg/ml mAb IV.3 followed by 30µg/ml $F(ab')_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218 for 20 minutes, or (D) 3µM TRAP6 amide for 10 minutes, before the collection of platelet-free supernatant and PF4 ELISA. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 in comparison to vehicle.

4.2.11 Platelet PF4 release in response to bacteria observed in some ibrutinib-treated CLL patients can be inhibited by FcγRIIa pathway inhibitors

Platelets derived from 4 ibrutinib-treated CLL patients aggregated in response to IV.3 XL and bacteria (Figure 4.2). For these samples, new aggregation reactions were performed (Figure 4.6) and platelet-free supernatants were collected and analysed by PF4 ELISA to assess α-granule release (Figure 4.9).

For all 4 donors for which this experiment was performed, *E. coli* RS218 had previously caused platelet aggregation. *S. aureus* Newman caused aggregation in two of the four donors, while IV.3 XL resulted in aggregation in one donor only. TRAP6 amide was used as a control.

As seen in Figure 4.9, all donors have a marked decrease in PF4 release when platelets stimulated with either IV.3 XL, *S. aureus* Newman, or *E. coli* RS218 were inhibited by either IV.3 mAb, dasatinib or ibrutinib compared to the vehicle, apart from donors 1 and 4 whose platelets when stimulated with *E. coli* RS218 were not inhibited by ibrutinib. This is very similar to the aggregation results in Figure 4.6, with a differentiation being in donor 3 ibrutinib inhibition response: there is a small inhibition of aggregation, but a greater decrease in PF4 release when compared to vehicle.

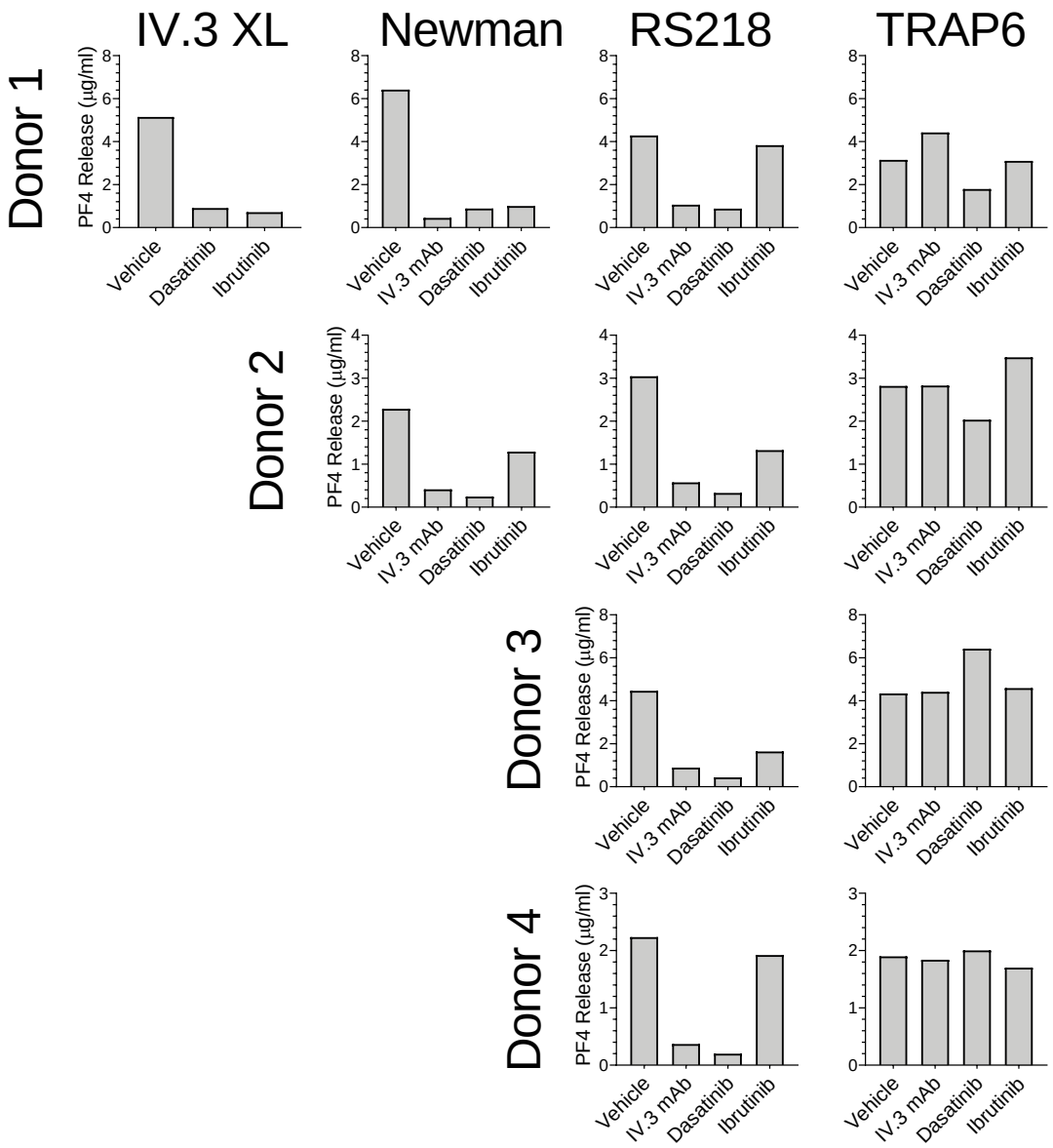


Figure 4.9. Bacteria-induced PF4 release from platelets derived from ibrutinib-treated CLL patients is FcγRIIa-dependent. In ibrutinib-treated CLL samples where platelet aggregation to bacteria was observed (see Figure 4.6) PRP was incubated with either vehicle, 20µg/ml mAb IV.3 , 4µM dasatinib or 5µM ibrutinib, before stimulation with either (A) 4µg/ml mAb IV.3 for 2 minutes followed by 30µg/ml $F(ab')_2$ rabbit anti-mouse IgG, (B) *S. aureus* Newman, (C) *E. coli* RS218 for 20 minutes, or (D) 3µM TRAP6 amide for 10 minutes before the collection of platelet-free supernatant and PF4 ELISA.

4.2.12 Ibrutinib-treated CLL patients' platelets are unable to scavenge bacteria

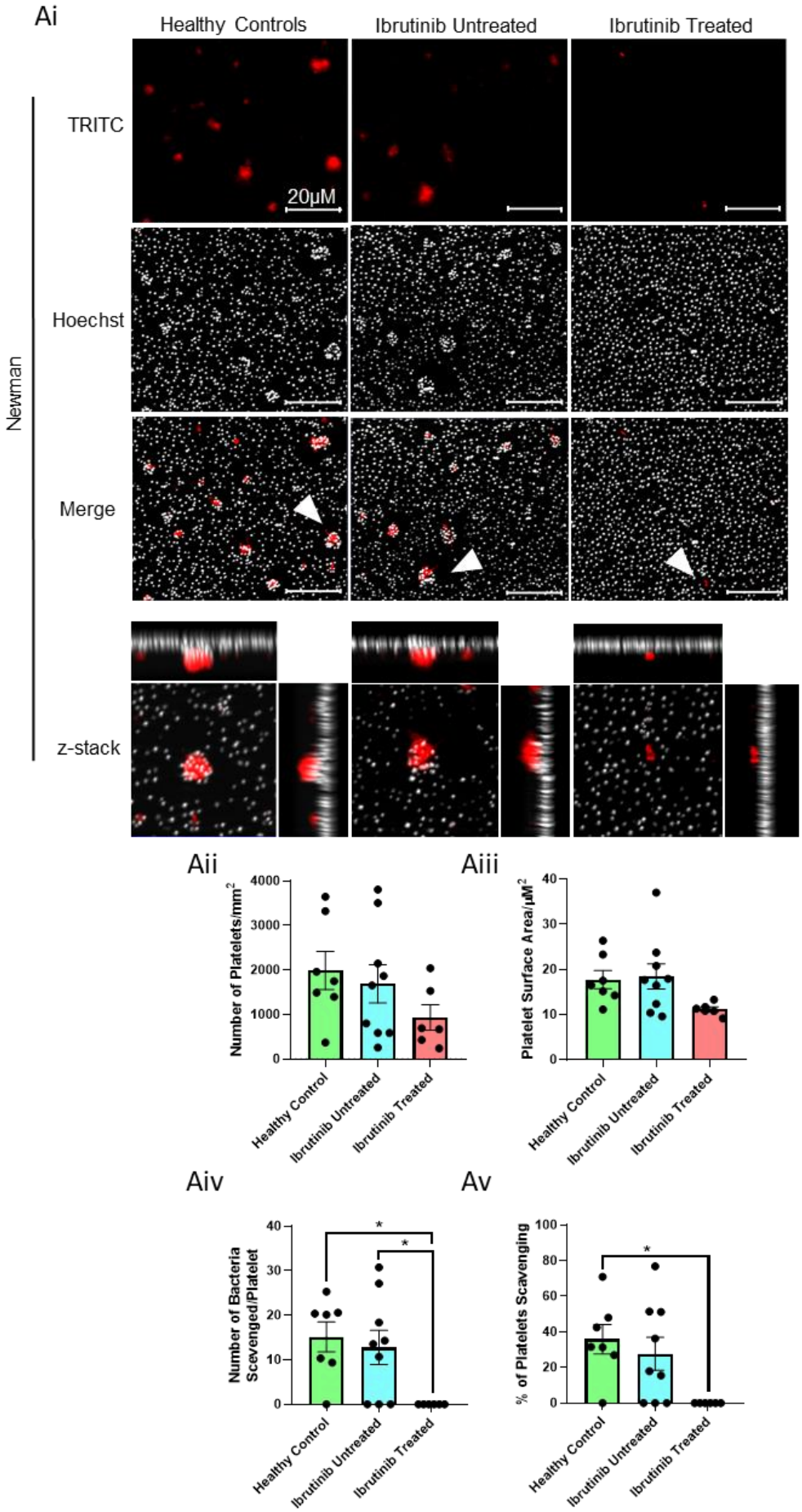
A previous study has analysed the ability of platelets to bundle fibrin-bound bacteria (Gaertner *et al.*, 2017). Platelets scavenging of bacteria represents an innate immune response. This platelet response is thought to aid phagocytosis due to the collection and localisation of bacteria, and increase of neutrophil activation (Gaertner *et al.*, 2017).

To evaluate whether ibrutinib treatment affects platelet scavenging of bacteria, a bacteria scavenging assay was performed. This assay compared platelet scavenging responses in healthy controls, ibrutinib-untreated and ibrutinib-treated CLL. Previously αIIbβ3 has been shown to be required for scavenging to take place (Gaertner *et al.*, 2017), and as such eptifibatide was used to inhibit αIIbβ3. This allowed for a comparison of the inhibitory effects between ibrutinib and αIIbβ3. Fibrinogen spreading was used as a control.

The results displayed in Figure 4.10 demonstrate a slight decrease in both the number of platelets adherent to the bacteria-coated surface, and in platelet surface area for the ibrutinib-treated CLL patients in comparison to healthy controls and ibrutinib-untreated CLL. Ibrutinib-treated CLL platelets were unable to scavenge bacteria in comparison to healthy controls (P=0.0205). Platelets derived from ibrutinib-untreated CLL had similar responses as healthy controls platelets in terms of the percentage of platelets scavenging and the number of bacteria scavenged per platelet (P=0.7362 and P=0.8573 respectively). Similarities can be seen between the z stacks of the healthy control and the ibrutinib-untreated CLL samples in terms of platelet size and pulling up of the bacteria from the bacteria layer. In contrast, the ibrutinib-treated CLL z stack shows the platelets as small with no changes in the bacteria layer. As expected, platelets in all three clinical groups did not spread or scavenge bacteria when treated with eptifibatide indicating that αIIbβ3 is required for these processes to take place. The ibrutinib-treated CLL z stack is comparable to the z stacks of the three clinical groups when treated with eptifibatide, indicating that inhibition by ibrutinib gives a similar phenotype as inhibition of αIIbβ3.

The fibrinogen control showed a decrease in adherence and platelet size of the ibrutinib-treated CLL platelets in comparison to the healthy control and ibrutinib-untreated CLL (Figure 4.10 C). The morphology of platelet spreading on fibrinogen was quantified into four categories: unclear, not spread, filopodia and lamellipodia. Lamellipodia is a wide projection from the platelet which contains a network of actin, whilst filopodia are finger-like projections from the platelet (Mattila and Lappalainen, 2008). All three clinical groups had similar levels of filipodia. Ibrutinib-untreated CLL and healthy control platelets had a similar pattern of morphologies with no significant differences found. However, ibrutinib-treated CLL platelets had lower levels of lamellipodia, and increased levels of non-spread platelets compared to healthy controls ($P<0.001$ and $P=0.003$ respectively).

Overall, ibrutinib-untreated CLL platelets had similar responses to healthy controls regarding bacteria scavenging and fibrinogen spreading and had the expected inhibition of bacteria scavenging when incubated with eptifibatide. This suggests that CLL patient platelets have normal bacteria scavenging responses. Compared to healthy controls, ibrutinib-treated CLL platelets a significant reduction in the number of platelets scavenging and a reduction of the number of bacteria bundled per platelet. Ibrutinib-treated CLL platelets have reduced adherence and surface area when spread on fibrinogen, with greater numbers not spread.


Ai
Healthy Controls
Ibrutinib Untreated
Ibrutinib Treated
TRITC
20μM
Hoechst
Merge
z-stack
Newman
Aii
Number of Platelets/mm2
4000
3000
2000
1000
0
Healthy Control
Ibrutinib Untreated
Ibrutinib Treated
Aiii
Platelet Surface Area/μM2
40
30
20
10
0
Healthy Control
Ibrutinib Untreated
Ibrutinib Treated
Aiv
Number of Bacteria Scevenged/Platelet
40
30
20
10
0
*
*
Healthy Control
Ibrutinib Untreated
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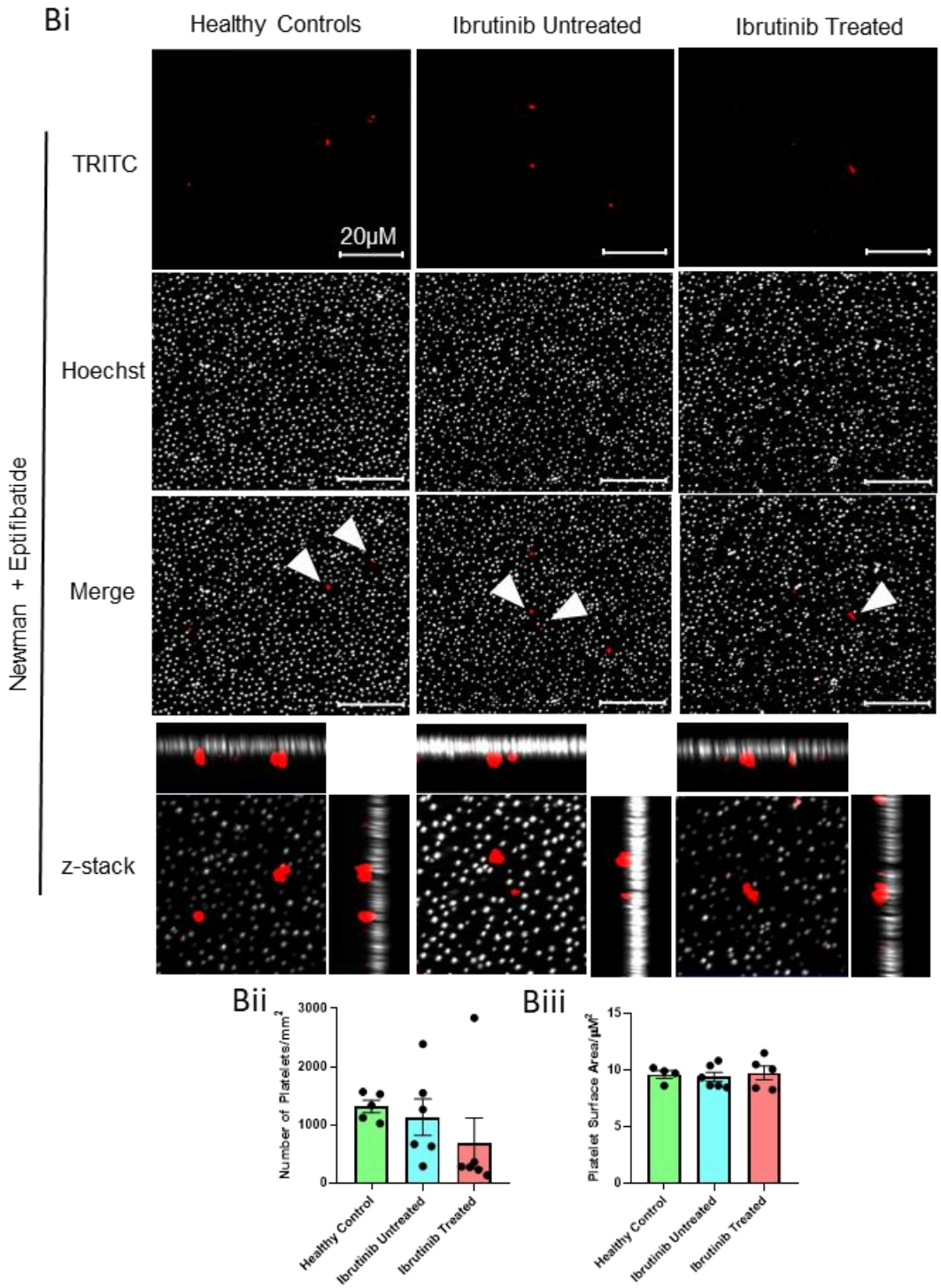
Bi
Healthy Controls
Ibrutinib Untreated
Ibrutinib Treated
TRITC
20μM
Hoechst
Merge
z-stack
Newman + Eptifibatide
Bii
Number of Platelets/mm²
3000
2000
1000
0
Healthy Control
Ibrutinib Untreated
Ibrutinib Treated
Biii
Platelet Surface Area/μM²
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Ibrutinib Treated

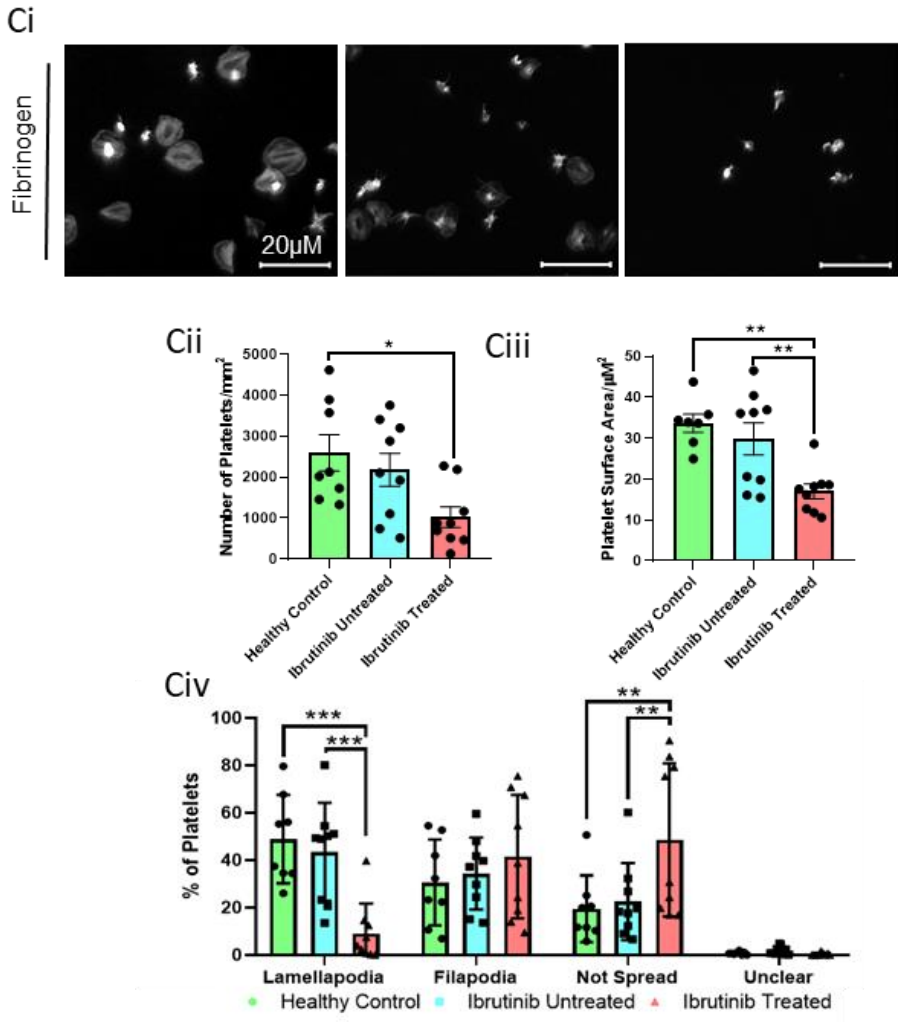


Figure 4.10. Ibrutinib-treated CLL platelets have reduced ability to scavenge bacteria. Platelets at 2x10^7 platelets/ml resuspended in 15% autologous plasma from healthy controls, ibrutinib-untreated CLL and ibrutinib-treated CLL patients were incubated with vehicle or 9µM eptifibatide and spread for 1 hour on *S. aureus* Newman or fibrinogen, fixed and then stained with TRITC phalloidin (for platelets) or Hoechst33342 (for bacteria). (A) Representative images and graphs pertaining to vehicle incubated platelets spread on *S. aureus* Newman which show number of platelets/mm^2, surface area of platelets, percentage of platelets scavenging, and number of bacteria per cluster. (B) Representative images and graphs pertaining to eptifibatide incubated platelets spread on *S. aureus* Newman showing number of platelets/mm^2 and surface area of platelets. (C) Representative images and graphs pertaining to vehicle incubated platelets spread on fibrinogen showing number of platelets/mm^2, surface area and morphology of platelets. In all images scale bar represents 20µm. Arrows on representative images indicate platelets shown in z-stack images. Data shows mean ±SD. *P<0.05 **P<0.01 ***P<0.001.

4.2.13 CD41 and GPVI Surface Expression is decreased in both CLL cohorts, but FcγRIIa levels remain the same

The previous results in this chapter show a difference in platelet responses to bacteria between healthy controls, ibrutinib-untreated and ibrutinib-treated CLL which could be due to changes in the surface expression of key receptors. A previous study (Dobie *et al.*, 2019) investigated platelet receptor levels after treatment with ibrutinib. The study showed that ibrutinib causes receptor shedding of GPIb-IX (a response thought to be mediated through ADAM17) and αIIbβ3. The study did not look at levels of FcγRIIa.

To ascertain if the CLL ibrutinib-treated cohort also displayed changes in receptor levels, flow cytometry was performed to assess αIIbβ3 (CD41), GPVI, and FcγRIIa (CD32) surface expression levels. A LSRFortessa cell analyser (BD Biosciences) was used to analyse PRP for platelet surface expression of receptors. CD41 was measured as it is a key receptor in platelet bacteria interactions through FcγRIIa (Arman *et al.*, 2014) (Watson *et al.*, 2016) and the aforementioned study found this receptor to have decreased platelet surface expression in response to ibrutinib (Dobie *et al.*, 2019). The study also found GPVI levels to remain the same. Another study, however, found decreased levels of GPVI in ibrutinib-untreated CLL platelets (Qiao *et al.*, 2013).

Figure 4.11 A demonstrates the gating strategy used to determine the platelet population based on CD32 and CD41 dual staining levels, as well as fluorescence levels for the non-stained, each antibody and the corresponding isotype control. 8-peak Rainbow Calibration Particles, allowing for standardisation against target reference values, were used in each experiment performed over the 6-week period, profiles of which are shown in Figure 4.12 C.

Figure 4.12 A shows the forward scatter (FSC) is similar among the three clinical groups, demonstrating that the size of the platelets is mainly unchanged in response to ibrutinib treatment. However, the side scatter (SSC), which is a readout of granularity, is decreased in platelets derived from ibrutinib-untreated CLL samples in comparison to both ibrutinib-treated CLL ($P<0.001$) and healthy control platelets ($P=0.001$). This possibly indicates that the platelets in ibrutinib-untreated CLL are preactivated and

hence degranulated, or it could indicate changes in megakaryocyte production. Figure 4.12 B shows that the levels of αIIbβ3 and GPVI are significantly decreased in both CLL groups compared to healthy control levels (αIIbβ3: P=0.003 and P<0.001 for ibrutinib-treated and ibrutinib-untreated versus healthy controls respectively. GPVI: P=0.003 and P=0.008 for ibrutinib-treated and ibrutinib-untreated versus healthy controls respectively). The reason for this is unknown but could relate to patient age or CLL diagnosis, though there is little published research into changes in platelet receptor levels during the aging process or in CLL patients. One study did rule out increased levels of metalloproteases being the cause for reduced GPVI levels in CLL as the plasma levels of GPVI was within a normal range (Qiao *et al.*, 2013). The previous study which demonstrated a reduced level of αIIbβ3 hypothesised this was down to an unknown shreddase in ibrutinib-treated CLL patients (Dobie *et al.*, 2019).

Figure 4.12 shows that FcγRIIa levels remain the same across the three groups (P=0.75 and P=0.97 for ibrutinib-treated and ibrutinib-untreated versus healthy controls respectively). This indicates that ibrutinib does not affect the surface levels of FcγRIIa, nor does the CLL condition. Hence, alterations in cell surface expression of the receptors measured in this study in response to ibrutinib do not account for the lack of platelet activation responses to FcγRIIa agonists including bacteria. However, there could be changes in surface expression of other platelet receptors both in CLL and due to use of ibrutinib.

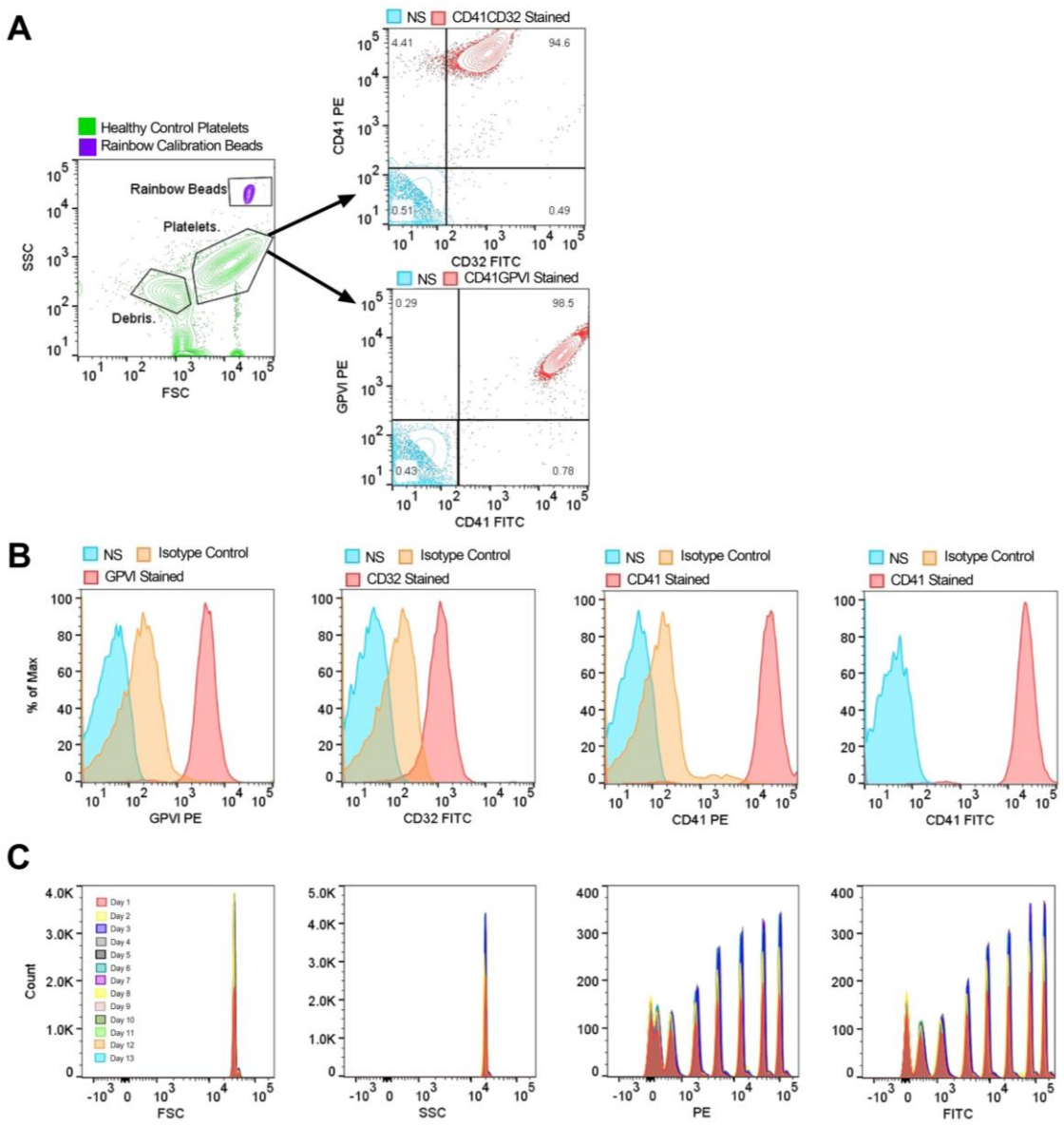
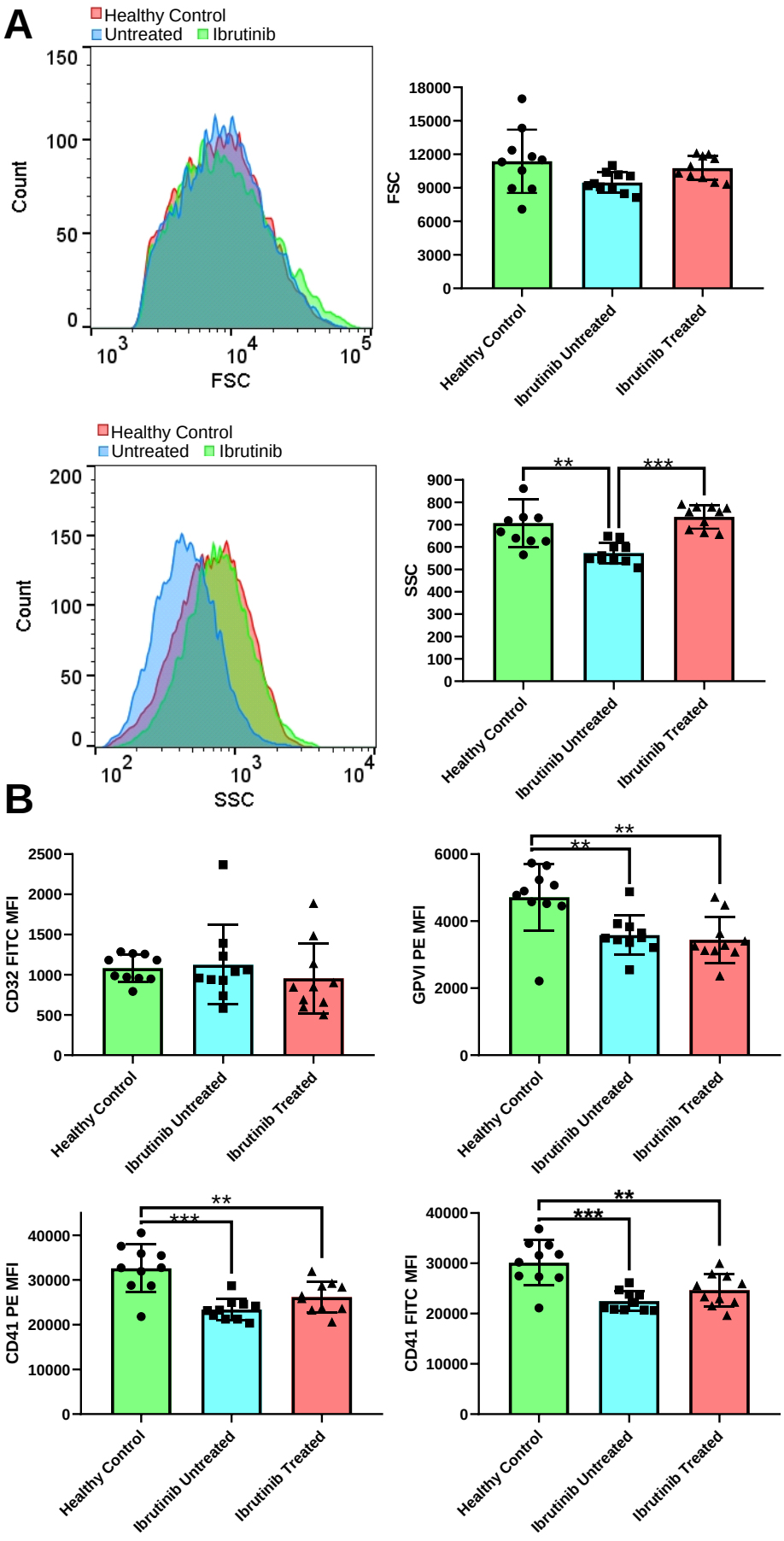


Figure 4.11. Gating strategy and experimental settings used in flow cytometry. (A) Left shows the SSC/FSC gating strategy for platelets in green with Rainbow Calibration beads (3.0-3.4 µm, BD Biosciences) detected separately in purple. Right depicts CD41-FITC/GPVI-PE dual stain and CD41-PE/CD32-FITC dual stain in red and non-stained cells in blue. (B) Comparison of antibodies, isotype controls and non-stained platelets. (C) Rainbow Calibration beads FSC, SSC, FITC and PE profiles from each of the 13 days flow cytometry assays were performed over six weeks.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Healthy Control
Untreated
Ibrutinib
Count
FSC
FSC
Healthy Control
Ibrutinib Untreated
Ibrutinib Treated
SSC
SSC
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***
B
CD32 FITC MFI
GPVI PE MFI
CD41 PE MFI
CD41 FITC MFI

Figure 4.12. Ibrutinib-treated and ibrutinib-untreated CLL platelets have lower surface expression of GPVI and αIIbβ3, while FcγRIIa remains comparable to healthy controls. Healthy donor, ibrutinib-untreated and ibrutinib-treated CLL PRP was diluted to $1x10^7$ platelets/ml and incubated with monoclonal antibodies against CD41 (αIIb subunit of human integrin αIIbβ3), GPVI or FcγRIIa (CD32) before flow cytometric analysis. (A) FSC and SSC of the platelets were determined. (B) Mean fluorescence intensities (MFI) of CD41, GPVI, and CD32 were measured. Two different antibody clones (PE-conjugated clone 5B12, FITC-conjugated clone P2) for CD41 were used as a technical replicate. Data is presented as mean ±SEM. *P<0.05, **P<0.01, ***P<0.001.

4.3 Discussion

The main finding from the studies above on the effect of ibrutinib on CLL patient platelet responses to bacteria and FcγRIIa crosslinking are:

- Ibrutinib-untreated CLL platelets respond to bacteria in the presence of autologous plasma.
- Ibrutinib-untreated CLL platelets have a decrease in granularity when compared to platelets obtained from ibrutinib-treated CLL patients and healthy controls.
- Ibrutinib-untreated and ibrutinib-treated CLL platelets have a decrease in surface levels of GPVI and αIIbβ3, but not FcγRIIa, when compared to healthy control platelets.
- Ibrutinib therapy impairs CLL platelet aggregation, scavenging and granule secretion in response to bacteria.

The results presented in this chapter are two-pronged: describing CLL FcγRIIa-dependent responses to bacteria, and then investigating the effect of ibrutinib on those responses.

However, the study of CLL patients must include consideration of a variety of other factors, namely comorbidities, co-medications, and the elderly patient group in comparison to the healthy controls. It is known from previous studies that many medications can affect platelet functions (Konkle, 2011), which was considered in this chapter. Well-known antiplatelet drugs include clopidogrel (adenosine diphosphate receptor inhibitor), and aspirin (inhibitor of cyclooxygenase) and were taken by 18% of ibrutinib-untreated CLL patients and 6% of ibrutinib-treated patients. Another anti-platelet drug targeting the ADP receptor, cangrelor, when used in combination with ibrutinib has been found to reduce thrombus stability to a greater extent than both inhibitors do individually (Bye *et al.*, 2015a). ADP receptor blockers and TXA_2 inhibitors, such as aspirin, inhibit the secondary mediators ADP and TXA_2 required for FcγRIIa-mediated platelet responses to bacteria (Arman *et al.*, 2014). As only a few patients in this study were on anti-platelet drugs, it is not possible to ascertain the effects of these

drugs, and their combination with iBtks, on CLL platelet responses to bacteria, though this would be an interesting area to explore in future research.

Patients from both the ibrutinib-untreated and ibrutinib-treated CLL cohorts were receiving a variety of concurrent medications. However, aggregation and PF4 release from ibrutinib-untreated CLL platelets was similar to healthy controls. Therefore, it could be presumed that medication and other CLL-specific factors have minimum effect on the platelet FcγRIIa pathway, and that all effects seen in aggregation, secretion and scavenging could be due to the ibrutinib treatment as well as any other inhibitor used in these studies. A previous investigation (Dmitrieva *et al.*, 2020) found that ibrutinib-untreated patients had decreased aggregation to ADP however this was not found in this current study. A limitation of the aforementioned study in this regard is only a few readouts of platelet activation (aggregation, flow cytometry for markers of platelet activation such as P-selectin, activated αIIbβ3) were measured and for a limited number of patients (n=50). This may mean that the effects could be different if a bigger cohort was tested, or there could be other platelet activation readouts affected which are not included in this study, such as dense granule release for the CLL patients.

Some drugs are known to affect platelet function, particularly: non-steroidal anti-inflammatory, selective serotonin reuptake inhibitors, antibiotics, and cardiovascular drugs (Scharf, 2012). Aggregation to TRAP6 amide is significantly decreased in ibrutinib-untreated CLL platelets. This could be due to a larger number of ibrutinib-untreated CLL patients being on statins, which have been shown to affect the PAR1 pathway, through which TRAP6 amide activates platelets (Serebruany *et al.*, 2006) (Fenton *et al.*, 2005) (Fenton *et al.*, 2002).

Increasing age is associated with a decrease in platelet count and platelet hyperactivity. The results from the studies presented in this chapter suggest that age does not play a major role in FcγRIIa-mediated platelet responses to *S. aureus* and *E. coli,* as well as in response to other agonists tested (CRP, TRAP6 amide and ADP), due to the similar degrees of platelet aggregation observed in healthy controls and ibrutinib-untreated CLL patients. However, high concentrations of agonists were used

here, while the age-related high sensitivity has been reported mostly at lower doses of agonist (Le Blanc and Lordkipanidzé, 2019). Also, lag times for aggregation in response to the two bacteria were quicker for ibrutinib-untreated CLL platelets in comparison to healthy controls, which could suggest platelet hyperactivity.

The levels and repertoire of IgGs in CLL patients are two other factors to consider when evaluating CLL platelet responses to bacteria due to the activation of FcγRIIa by IgG opsonised bacteria. CLL is associated with hypoagammaglobulinaemia, and reduced levels of IgA at diagnosis is a predictor for infection risk, survival and time to first treatment in CLL (Ishdorj *et al.*, 2019). Moreover, numerous studies have shown deficiencies in IgG are frequent. Age can affect immunoglobulin isotype levels and immunoglobulin concentrations with IgG increasing until teenage years, before plateauing (Ritchie *et al.*, 1998), with other studies suggesting that levels decrease after 70 years of age (Lock and Unsworth, 2003).

Some CLL patients in this study could have hypoagammaglobulinaemia which could alter the interactions of FcγRIIa with bacteria. This is supported by one ibrutinib-untreated CLL patient in which addition of IgG allowed for platelet aggregation in response to *E. coli* RS218 to commence. However, overall, CLL platelet aggregation and PF4 release appear to be normal. This suggests that hypoagammaglobulinaemia is not a major factor controlling FcγRIIa-mediated platelet responses to bacteria in CLL, however, this cannot be concluded without measuring IgG levels and types in patients at time of sample collection.

It was previously unknown if platelets from CLL patients had the ability to activate in response to bacteria. The results presented herein show that platelets derived from ibrutinib-untreated CLL patients respond to both encapsulated gram positive (*S. aureus* Newman (Wang *et al.*, 2018)) and gram negative (*E. coli* RS218 (Xie *et al.*, 2006)) bacteria in a similar manner to that observed in healthy controls. CLL patients are more prone to infection via encapsulated bacteria (Wadhwa and Morrison, 2006) (Tsiodras *et al.*, 2000), though *Streptococcus pneumoniae* and *Haemophilus influenza* are the most frequent causes of infection (Wadhwa and Morrison, 2006). This data suggests that during a bloodborne infection, platelets from CLL patients can respond to

encapsulated bacteria, with resultant aggregation and granule release contributing to the overall immune response. However, some CLL platelets did not fully activate to bacteria, and as such could have defects or changes in the platelets due to various reasons mentioned previously surrounding CLL, which could then be worsened by ibrutinib treatment. Although this investigation focused on *S. aureus* Newman and *E. coli* RS218, other bacterial strains and species, including *Streptococcus pneumoniae* and *Haemophilus influenza,* should also be studies in this context.

In addition to the inhibitory effect of ibrutinib therapy, this study demonstrated that platelet aggregation and PF4 release in CLL samples stimulated with bacteria were mediated through the FcγRIIa pathway as evidenced by the inhibitory effects of FcγRIIa pathway inhibitors, IV.3 mAb and dasatinib. However, in a few ibrutinib-treated CLL patients, aggregation and PF4 release still occurred in response to bacteria, particularly *E. coli* RS218, which could not be inhibited by further addition of ibrutinib *in vitro* but was inhibited by IV.3 mAb and dasatinib, raising the question of the role of Btk in platelet activation by bacteria.

Different strains of *E. coli* can interact with platelets in numerous ways which do not require the FcγRIIa receptor, such as TLR4 (Matus *et al.*, 2017), however, *E. coli* RS218 is known to work through FcγRIIa without TLR4 involvement (Watson *et al.*, 2016). However, there could be other unexplored pathways which *E. coli* RS218 is utilising to activate platelets that are not affected by ibrutinib. Another possible reason for the inability of ibrutinib to inhibit platelet aggregation in response to bacteria is the possible involvement of Tec in the pathway. Previous studies have investigated the role of Btk and Tec, predominantly in the GPVI signalling pathway, and suggested that these kinases may have redundant functions (Atkinson, Ellmeier and Watson, 2003b) It is possible that Tec could also be playing a compensatory role within the FcγRIIa pathway, allowing the reactions to still occur. However, investigation into platelet Btk and Tec phosphorylation downstream of FcγRIIa in response to bacteria, and the effect of ibrutinib on this, is needed.

Another way in which platelets mediate innate immunity has been studied in which platelets can migrate and scavenge fibrin(ogen) and including bacteria bound to fibrin

(Gaertner *et al.*, 2017). The ability of platelets to scavenge from their microenvironment is dependent upon actomyosin forces with pathogens being attached to the surface of the platelet. The structure of platelets with bacteria attached to their surface has been described as similar to bacterial biofilms. Physiologically, platelets are recruited to infection sites where they can scavenge bacteria. In addition, platelets can recruit and activate other immune cells. This platelet-mediated collecting of bacteria could potentially aid the other immune cells in the clearance of bacteria. As such, exploring this mechanism was an important part of this study.

Regarding the bacteria scavenging assays performed in this study, platelets from ibrutinib-treated and ibrutinib-untreated CLL patients showed similar adherence to *S. aureus* Newman, but scavenging was completely inhibited in the former. Though the mechanism behind platelet scavenging is not fully understood, FcγRIIa is frequently required for platelet interactions with *S. aureus* (Cox, Kerrigan and Watson, 2011), while αIIbβ3 is required for scavenging of fibrin-bound bacteria (Gaertner *et al.*, 2017). Btk lies downstream of both FcγRIIa and αIIbβ3 signalling. Btk is upstream of PLCγ2, the activation of which causes the inside-out activation of αIIbβ3 as well as the activation of Rho-GEFs which are required for cytoskeletal rearrangements. Ibrutinib is known to affect both FcγRIIa and αIIbβ3 signalling, therefore the inhibition of scavenging observed could be due to the effect of ibrutinib on both receptors.

Scavenging assays were done in the presence of plasma, thus including fibrinogen and IgGs that are ligands for αIIbβ3 and FcγRIIa respectively. *S. aureus* Newman expresses proteins, namely ClfA and B, known to bind to fibrinogen (Cox, Kerrigan and Watson, 2011), which is important for inducing platelet aggregation through the cooperative action of αIIbβ3 and FcγRIIa. Future studies should be done to determine the relative contribution of fibrinogen and αIIbβ3 and IgGs and FcγRIIa on platelet spreading of S. aureus Newman and other bacteria. This could be done by using washed platelets supplemented with fibrinogen plus and minus IgGs, or IgG-depleted PRP. The use of *S. aureus* Newman mutants, in which specific proteins like ClfA/B, are deleted to determine the effects of *S. aureus* binding to fibrinogen could be used too. Overall, the

assays performed in the present study demonstrated that ibrutinib causes an impairment in platelet-mediated scavenging.

In the scavenging assays, platelets of healthy controls and ibrutinib-treated patients are seen to be larger than those of Integrilin treated counterparts spread on *S. aureus* Newman. Average platelet size of eptifibatide-treated platelets which are not activated was around 10µm^2, while for bacteria spread platelets it was around 20µm^2, and for fibrinogen spread platelets which are activated it was around 30µm^2. This suggests that bacteria-scavenging platelets are activated due to the increase in size from the eptifibatide-treated platelets. This activation may be to a similar extent as that seen for fibrinogen, but due to the hypothesis that platelets spread their membranes over pathogens and then contract to encapsulate (Gaertner *et al.*, 2017) this could explain the smaller size. This hypothesis that platelets are activated when spread on bacteria is likely as this change in size and shape is demonstrated in another study from our group in which the inhibition of actin polymerisation via latrunculin prevented platelet scavenging of *S. aureus* Newman (Chacko, 2020). Different platelet spreading phenotypes have been demonstrated in a previous study (Lickert *et al.*, 2018) in which it was shown that platelets spread on fibrinogen, fibronectin, and collagen had similar shapes, while platelets spread on laminin were rounder. Platelets spread on fibrinogen, fibronectin and laminin were a similar size at 30µm^2, while those spread on collagen were around 20µm^2. Other studies have shown that morphological shape change of platelets can be dependent on the function the platelet is performing. As such, platelets carrying out a haemostatic function stimulated with thrombin show long thin pseudopodia with greater numbers in platelet clusters compared to when stimulated through a virus which activates TLR7 or a TLR7 ligand for an immune response, which instead results in fewer and shorter pseudopodia, smaller clusters of platelets and interactions with leukocytes (Koupenova *et al.*, 2018). This shows that platelets spread on different matrixes are able to have different phenotypes, and as such the phenotype seen in this study of platelets spread on bacteria may be due to the matrix of bacteria and has previously been seen in another study (Chacko, 2020).

Platelets in the scavenging assays were able to show attachment to bacteria which were otherwise attached to the coverslip via poly-L-lysine without shear. This has

previously been demonstrated in other studies (Chacko, 2020) which have also shown that over the course of an hour the number of bacteria scavenged by bacteria significantly increases from 15 minutes to 30 minutes, and from 30 minutes to an hour. Majority of studies using pol-L-lysine to so at a concentration of 0.01% (Colville *et al.*, 2010) (Aliko *et al.*, 2018) (Muniyandi *et al.*, 2020), while in this study the concentration was at 0.005% so it is possible that this lower concentration allows for easier removal of the bacteria from the coverslip by the platelets. Previous studies (Gaertner *et al.*, 2017) have also shown that platelets are able to migrate on fibrinogen with *E. coli* against the direction of flow even at arterial flow velocities, making it possible that platelets are able to migrate in the current study with no shear when scavenging bacteria. The effects of shear on platelet scavenging should be further explored.

The flow cytometry data from this chapter interestingly shows a decrease in granularity in ibrutinib-untreated CLL platelets in comparison to both healthy controls and ibrutinib-treated CLL. This decrease in granularity could be due to platelet activation, possibly due to increased levels of B cells in the blood or another activating molecular or cellular factor in the blood of the CLL patients, or it could also be due to changes in the bone marrow and megakaryocyte production. This level of platelet activation seems to be restored with ibrutinib treatment.

In this study, expression of FcγRIIa on the platelet surface in the clinical groups was taken into consideration. FcγRIIa is expressed at different levels from donor to donor (Tomiyama *et al.*, 1992) (Rosenfeld *et al.*, 1987), with the levels of individual healthy donors remaining consistent. FcγRIIa expression levels can be altered in some diseases such as heparin-induced thrombocytopenia (Chong *et al.*, 1993), and increased expression levels on platelets are a risk factor for certain diseases such as cardiovascular disease due to increased platelet reactivity (Serrano *et al.*, 2007) (Liebeskind *et al.*, 2021). Ibrutinib has previously been reported to exert off-target effects on platelet surface receptors expression. One study (Dobie *et al.*, 2019) found that ibrutinib did not affect levels of GPVI or GPV but did cause the shedding of αIIbβ3 and GPIb-IX, the latter through an a disintegrin and metalloprotease 17 (ADAM-17) dependent mechanism in both healthy controls *in vitro* as well as CLL patients. In the studies presented herein, there was no change identified in FcγRIIa surface expression

levels among healthy controls, ibrutinib-untreated and ibrutinib-treated CLL patients, allowing the conclusion that ibrutinib has no major effect on FcγRIIa surface expression.

FcγRIIa has two polymorphisms at amino acid 131: one histidine and one arginine allotype. The histidine allotype has a greater binding affinity for IgG_2 and IgG_3 (Warmerdam *et al.*, 1991). The polymorphisms are associated with increased risk of infectious and autoimmune diseases as well as other diseases (Sanders *et al.*, 1995) (Kyogoku *et al.*, 2002) (Mkaddem, Benhamou and Monteiro, 2019). The distribution of FcγRIIa polymorphisms within each clinical group included in this study may be uneven, and may contribute to the results observed, however, polymorphisms were not tested.

In contrast to FcγRIIa, a decrease in both αIIbβ3 and GPVI was seen in both groups of CLL patients in comparison to healthy controls. This would suggest that ibrutinib is not affecting these receptors, and such down regulation may be secondary to CLL itself or patient age which is known to affect platelet structure (Le Blanc and Lordkipanidzé, 2019). The lower levels of αIIbβ3 could have played a role in the decreased levels of scavenging of bacteria. Results of receptor levels do differ from previous studies (Dobie *et al.*, 2019) in which, here, receptor levels changed only in platelets from ibrutinib-treated CLL patients. One possible reason for this discrepancy could be due to difference in sample collection, with healthy samples being collected on site, while CLL samples were collected in vacutainers in Castle Hill Hospital. Moreover, the current study was performed in PRP rather than washed platelets as done in previous investigations, and substances in the plasma may be present which could affect receptor cleavage. However, both in this study and in others, only a small number of patients were used in investigations and as such cannot be counted as representative of a population. As such, more exploration is needed to distinguish between sample collection and preparation or a CLL-specific factor in platelet receptor levels.

A previous study (Series *et al.*, 2019) found platelets from different donors have differing sensitivity to ibrutinib based on drug efflux pumps, with inhibition of these pumps resulting in increased sensitivity to ibrutinib. However, testing with the iBtks

was performed *in vitro* with no *in vivo* data. The current study was limited to ibrutinib-treated CLL platelets due to very low numbers of CLL patients receiving acalabrutinib therapy in our local area during the duration of the sample recruitment. Future exploration of ibrutinib-sensitivity for the FcγRIIa pathway in platelets could be explored and the possible impact this could have on patients, in addition to the effects *in vivo* treatment with acalabrutinib on platelet responses to bacteria.

Overall, the results of this study suggest that platelets from CLL patients do respond to bacteria, but these responses are impaired by use of ibrutinib, which in turn could increase the risk of infection observed in these patients. Ibrutinib treatment in patients has inhibitory effects in other immune cells. As such, future studies could investigate the relative effect of ibrutinib on different immune cells including platelets, as well as the effect on the interactions among them, and how this could increase the risk of infection.

Chapter 5: The Effects of Ibrutinib and Acalabrutinib on Btk Activation in FcγRIIa Mediated Platelet Activation in Response to Bacteria

5.1 Introduction

Ibrutinib and acalabrutinib target cysteine 481 in the Btk ATP binding region, hence blocking kinase activity thereby inhibiting B cell proliferation. Btk C481 is a nucleophilic site which was chosen as a therapeutic target due to its potential to form a covalent complex with an electrophilic compound, such as ibrutinib. The sequences of 491 kinases were aligned, and out of this, 10 kinases were found to contain a cysteine at the same position, including Btk and Tec (Pan *et al.*, 2007).

For Btk activation, Syk phosphorylates Btk at Y551, which results in the autophosphorylation of Y223 and the full activation of Btk. Previous studies in platelets found that ibrutinib and acalabrutinib block a signal through the GPVI pathway resulting in normal pY551 phosphorylation but impaired pY223 phosphorylation (Nicolson *et al.*, 2018a). The same study found that ibrutinib and acalabrutinib inhibit the phosphorylation of Tec at around 3 times the concentration of drug needed to block Btk in washed platelets. In addition, it was found that inhibition of GPVI aggregation by ibrutinib required 10-20 times more ibrutinib than that required to completely inhibit Y223 phosphorylation of Btk.

This excess of ibrutinib required to inhibit GPVI aggregation after Btk inhibition is suggestive of off-target effects, predicted to be the effect of ibrutinib on Tec, as well as other kinases such as Src. Using knockout mice models, Tec has been found to substitute for Btk in the GPVI pathway in the absence of Btk (Atkinson, Ellmeier and Watson, 2003b). In addition, in XLA patients who lack functional Btk, Tec compensates for Btk in GPVI signalling (Nicolson *et al.*, 2018a). Studies utilising platelets from XLA patients demonstrate ibrutinib can inhibit aggregation of XLA platelets to CRP at similar concentrations which would otherwise inhibit Btk (Nicolson *et al.*, 2018a).

Many studies have since found ibrutinib to have off-target effects on both Tec and Src kinases (Bye *et al.*, 2017) (Series *et al.*, 2019) (Nicolson *et al.*, 2018a).

FcγRIIa is an ITAM receptor with one previous study demonstrating phosphorylation of both Tec and Btk in response to crosslinking of the FcγRIIa (Oda *et al.*, 2000), however, it is unknown if Btk is phosphorylated in response to FcγRIIa activation by bacteria. One study has previously inhibited antibody-mediated FcγRIIa crosslinking by the use of iBtks (Goldmann *et al.*, 2019) and suggested that Tec was not involved in this process. However, it is unknown if Btk is activated or essential in the platelet's aggregatory responses to bacteria, and if iBtks affect this either through Btk or through off-target effects on other proteins.

Hypothesis:

The hypothesis investigated in this chapter is: Btk is activated upon FcγRIIa-mediated platelet interaction with bacteria, and such activation is inhibited by ibrutinib and acalabrutinib.

Aims:

The questions to be answered in this chapter are:

- Are platelet Btk and Tec activated in response to bacteria?
- Is Btk essential in the FcγRIIa-mediated platelet response to bacteria?
- How do ibrutinib and acalabrutinib modulate platelet intracellular signalling in response to bacteria?

To answer the first and third questions, lysates were collected from PRP samples derived from healthy donors and CLL patients upon stimulation with IV.3 XL and bacteria, which were analysed for total Btk and pY223 Btk by western blotting.

To establish if Btk is essential for FcγRIIa mediated response to bacteria, LTA was performed for XLA derived platelets in the presence of plasma and stimulated with crosslinked IV.3 mAb and bacteria, as well as TRAP6 amide, CRP, and ADP. Upon platelet aggregation being detected, further inhibitory conditions were investigated as determined in chapter 3 to establish if the FcγRIIa pathway was still functional.

To investigate if Tec is phosphorylated in platelets in response to bacteria and subsequently the effects of *in vivo* and *in vitro* ibrutinib, lysates were collected from washed platelets derived from healthy control, ibrutinib-treated and ibrutinib-untreated CLL patients upon stimulation with various agonists and a Tec phosphorylation ELISA was performed.

5.2 Results

5.2.1 Platelet Btk is phosphorylated downstream of FcγRIIa and inhibited in ibrutinib-treated CLL patients

As it was unknown if Btk is downstream of FcγRIIa activation by IgG-opsonised bacteria, western blotting was carried out with PRP derived platelet lysates of healthy controls and ibrutinib-untreated CLL patients stimulated with CRP, IV.3 XL, TRAP6 amide, *S. aureus* Newman and *E. coli* RS218 (Figure 5.1). This figure shows an increase in phosphorylation of Btk pY223 for all agonists tested in comparison to basal conditions, with the highest levels of phosphorylation detected for CRP stimulated platelets. Phosphorylation to FcγRIIa agonists is notably around 2-fold lower than phosphorylation due to CRP. Similar patterns of phosphorylation are detected between ibrutinib-untreated CLL platelets and healthy donors.

To investigate the effect of ibrutinib therapy on platelet Btk activation, platelet samples were obtained from ibrutinib-treated CLL patients and western blotting for pY223 Btk was performed (Figure 5.1). As expected, phosphorylation of Y223 Btk is abolished compared to the healthy control samples run in parallel.

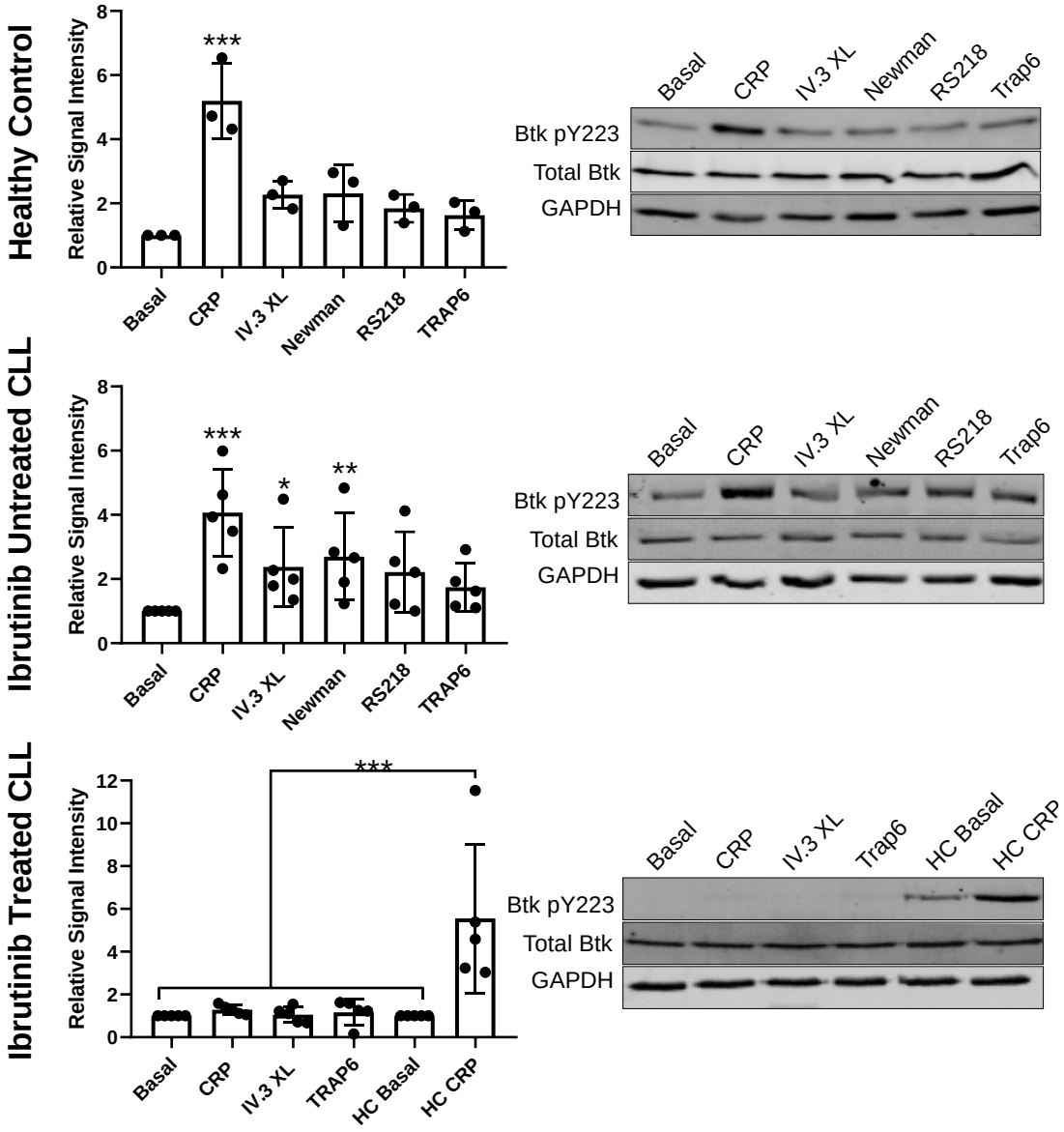
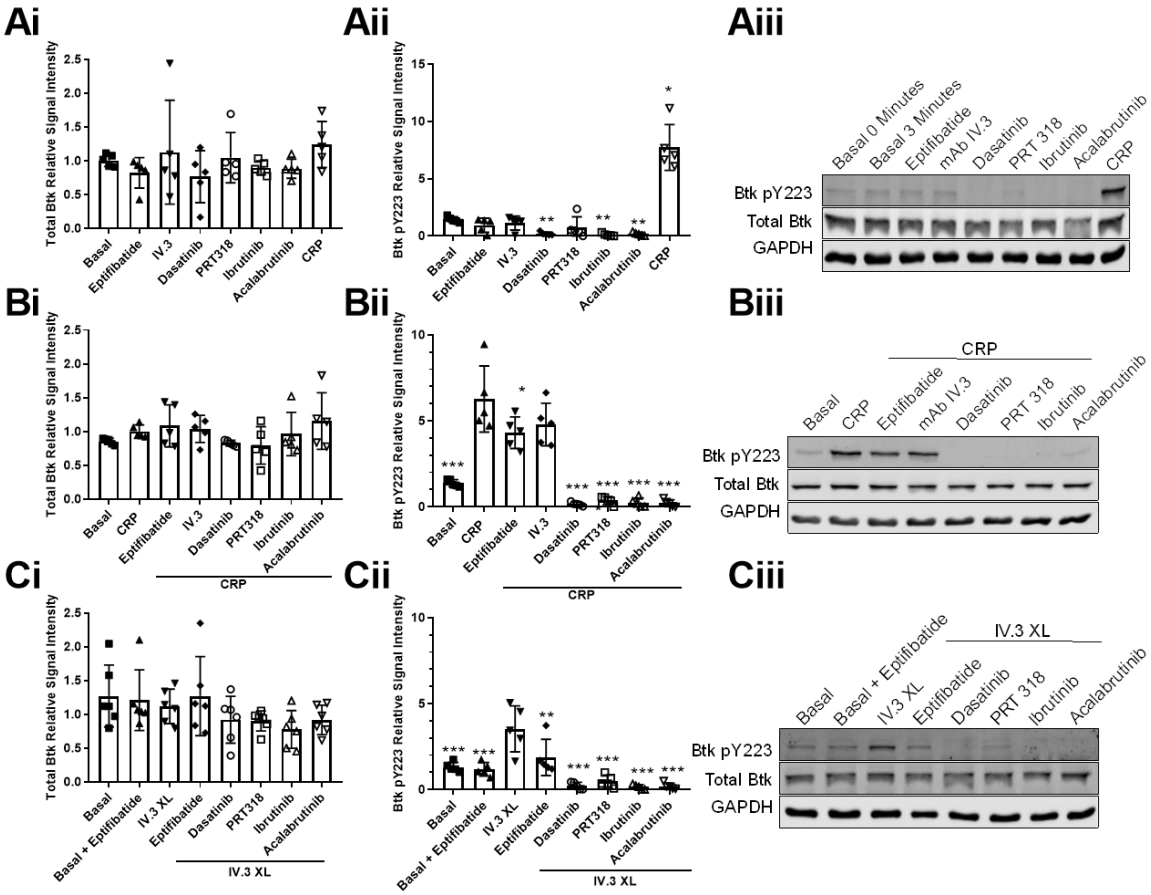


Figure 5.1. Stimulation of platelets by IV.3 crosslinking and bacteria results in the phosphorylation of Btk in healthy controls and ibrutinib-untreated CLL patients but is not seen in ibrutinib-treated CLL platelets. PRP from healthy donors, untreated CLL and ibrutinib-treated CLL patients was stimulated with either 3µg/ml CRP, 4µg/ml mAb IV.3 followed by 30µg/ml $F(ab')_2$ rabbit anti-mouse IgG, *S. aureus* Newman, *E. coli* RS218 or 3µM TRAP6 amide. Lysates equivalent to $5x10^8$ platelets/ml were collected 3 minutes after the start of aggregation and immunoblotted for pY223 Btk and total Btk. Figure shows pooled data (left) and representative blots (right). pY223 Btk was normalised against GAPDH. HC=healthy control. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 where ibrutinib-untreated and healthy controls are compared with basal conditions.

5.2.2 Btk Y223 phosphorylation is inhibited by a range of FcγRIIa pathway inhibitors

To investigate ibrutinib and acalabrutinib inhibition of Btk activation, PRP was incubated with either 5µM ibrutinib or 15µM acalabrutinib before stimulation with either PBS, CRP, IV.3 XL, *S. aureus* Newman, *E. coli* RS218 or TRAP6 amide. To compare the levels of inhibition exerted on Btk by ibrutinib and acalabrutinib, the inhibitors IV.3 mAb, dasatinib, eptifibatide and PRT-318 were compared alongside. The results in Figure 5.2 show that pre-treatment of platelets with IV.3 mAb and eptifibatide returns Btk Y223 phosphorylation in response to bacteria to levels seen in basal conditions. However, use of dasatinib, PRT-318, ibrutinib and acalabrutinib inhibits Btk phosphorylation to levels below that seen for basal samples, almost removing signal completely, though dasatinib and PRT-318 would be presumed to inhibit upstream proteins Src and Syk to prevent Btk activation.



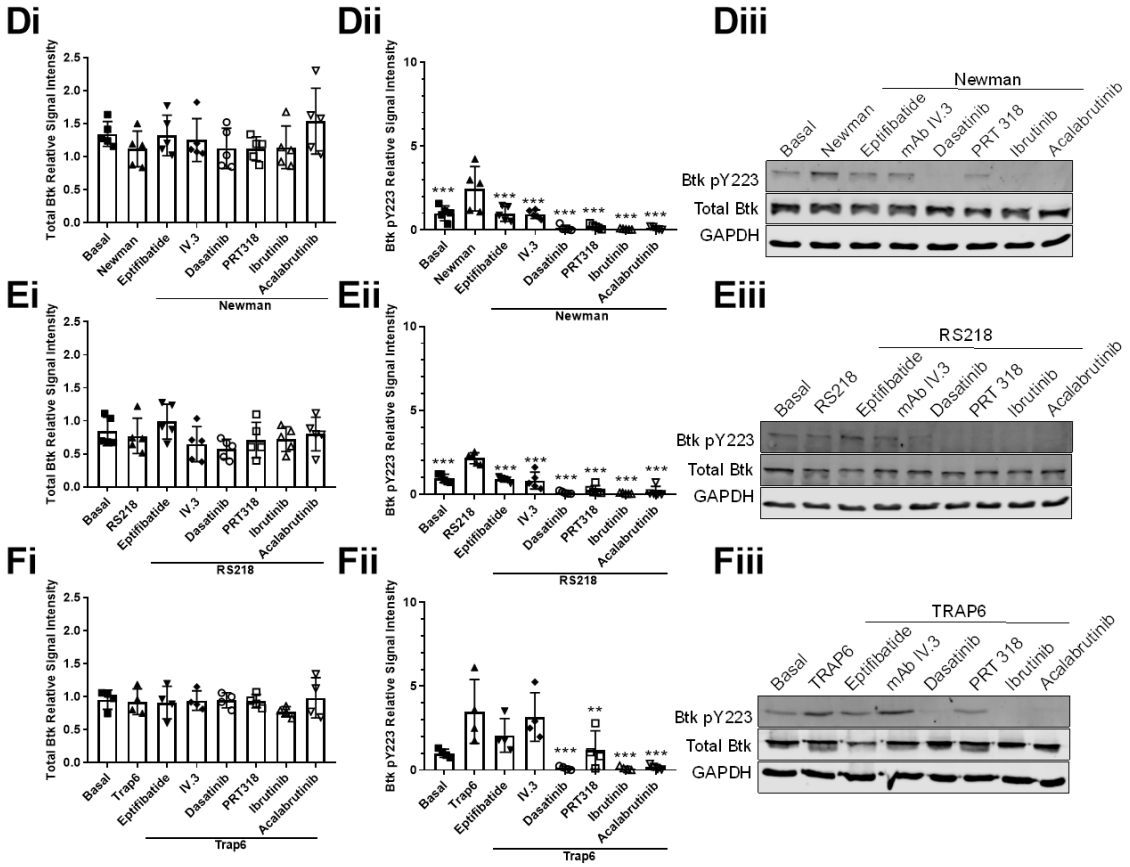


Figure 5.2. Btk phosphorylation is inhibited by a range of FcγRIIa pathway inhibitors. Healthy donor PRP was incubated with vehicle, 20µg/ml IV.3 mAb, 4µM dasatinib, 10µM PRT-318, 9µM eptifibatide, 5µM ibrutinib or 15µM acalabrutinib before stimulation with (A) vehicle, (B) 3µg/ml CRP, (C) 4µg/ml mAb IV.3 followed by 30µg/ml $F(ab')_2$ rabbit anti-mouse IgG, (D) *S. aureus* Newman, (E) *E. coli* RS218, (F) 3µM TRAP6 amide. Lysates at $5x10^8$ platelets/ml were collected 3 minutes after the start of aggregation or a parallel time point in samples in which aggregation was inhibited and blotted for either (i) total Btk (D3H5) or (ii) phosphor Btk (pY223) with representative blots shown in (iii). Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001 compared to agonist alone.

5.2.3 Btk Y223 phosphorylation is inhibited at a lower dose of ibrutinib than aggregation

Other studies focused on GPVI have reported inhibition of pY223 Btk at a lower concentration of ibrutinib than that needed to inhibit aggregation, suggesting that the concentration of ibrutinib required to inhibit platelet aggregation results in the inhibition of kinases other than Btk. To evaluate if the same effect is seen in the FcγRIIa signalling pathway by ibrutinib, further western blotting was carried out to test aggregation versus Btk pY223 signal at time of lysis when PRP was stimulated with IV.3 XL or CRP and inhibited with increasing concentrations of ibrutinib (Figure 5.3).

A concentration of 1µM ibrutinib was able to reduce levels of pY223 Btk to basal levels when stimulated with IV.3 XL, while concentrations of 2µM and above were able to decrease levels of pY223 Btk below basal levels. 1µM of ibrutinib and above was able to partially decrease platelet aggregation, with 5µM ibrutinib resulting in complete inhibition of aggregation.

When CRP was used to stimulate the GPVI pathway, there was no tendency of reducing levels of aggregation as Btk pY223 signal decreased. 1µM and above of ibrutinib was sufficient to decrease pY223 Btk levels when stimulated with CRP to basal levels, while 5µM ibrutinib was able to reduce pY223 levels to below basal levels. However, 5µM of ibrutinib was not able to inhibit platelet aggregation in response to CRP.

Although inhibition of Btk pY223 (at detectable levels) did not correlate with a complete inhibition of IV.3 XL induced aggregation, results from Figure 5.3 suggest that the FcγRIIa signalling pathway is more sensitive to ibrutinib, and potentially more dependent on Btk, than the GPVI signalling pathway in healthy donor platelets.

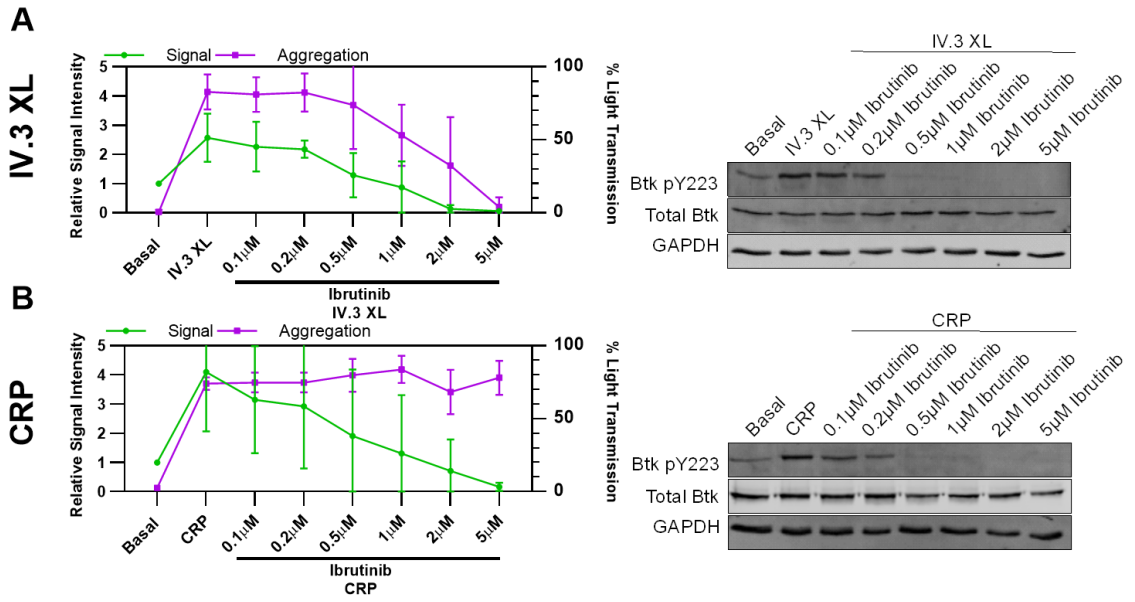


Figure 5.3. Btk Y223 phosphorylation is inhibited at lower concentrations of ibrutinib than that needed to block aggregation in response to IV.3 XL. PRP was incubated with vehicle or specified concentrations of ibrutinib for 5 minutes before the addition of either (A) 4μg/ml mAb IV.3 followed by 30μg/ml $F(ab')_2$ rabbit anti-mouse IgG, or (B) 3μg/ml CRP. Reactions were monitored by LTA and were stopped at 3 minutes after the start of aggregation to prepare lysates equivalent to $5x10^8$ platelets/ml for western blotting. Graphs on the left-hand side show aggregation at time of lysis versus p223 Btk signal, while representative western blots are shown on the right. Data shows mean ±SD.

5.2.4 XLA platelets can aggregate in response to a range of bacteria

XLA platelets are known to aggregate in response to CRP and collagen despite having non-functional Btk (Nicolson *et al.*, 2018a). Moreover, XLA patients do not show a bleeding phenotype (Quek, Bolen and Watson, 1998), unlike ibrutinib-treated CLL patients. This has been proposed to be due to Tec substituting for Btk to maintain platelet function. To investigate if platelets can respond to bacteria in an FcγRIIa-dependent manner in the absence of functional Btk, XLA PRP derived from two patients (one repeated on three separate days) were stimulated with a range of bacteria including *S. aureus* Newman, *E. coli* RS218, *E. coli* CFT073, *S. sanguinis* 133-79, *S. gordonii* DL1, and *S. oralis* CR834, which are all known to activate platelets in an FcγRIIa-dependent manner (Arman *et al.*, 2014) (Watson *et al.*, 2016).

As shown in Figure 5.4, platelets derived from both XLA patients responded to all bacteria used except for *S. gordonii* DL1 in the case of one patient. In both patients there is a poor aggregatory response to IV.3 XL. Results from Figure 5.4 C confirm that the FcγRIIa pathway is utilised in both XLA patients as evidenced by the ability of IV.3 mAb to inhibit platelet aggregation in response to bacteria.

These results demonstrate that Btk is not required for FcγRIIa-mediated platelet aggregation in response to bacteria, though suggests that activation of FcγRIIa by IV.3 XL is more sensitive to loss of Btk than when the receptor is activated by bacteria. As Tec is known to substitute for Btk in the GPVI pathway, it is possible that Tec is also substituting, at least partially, for the loss of Btk function in the FcγRIIa pathway.

Table 5.1. Medication types XLA patients were on at sample collection. Concurrent medications XLA patients were on during the study was categorised by drug type based on the British National Formulary (Joint Formulary Committee. (2018). BNF 76th Ed. Pharmaceutical Press).

Patient	Drug Type	Drug
Patient 1	Intravenous Immunoglobulins	Gamunex 10% intravenous immunoglobulin
	Antibiotic	Azithromycin, Nebulised Tobramycin
	Corticosteroids	Symbicort
	Bronchodilator	Nebulised ipratropium bromide, Tiotropium bromide, Salbutamol, Nebulised Salbutamol
	Antidepressant	Citalopram
	Vitamins	Colecalciferol (vitamin D)
Patient 2	Intravenous Immunoglobulins	Hizentra 20% subcutaneous immunoglobulin (IgG only)
	Antibiotic	Erythromycin
	Anti-Epileptic	Sodium valproate

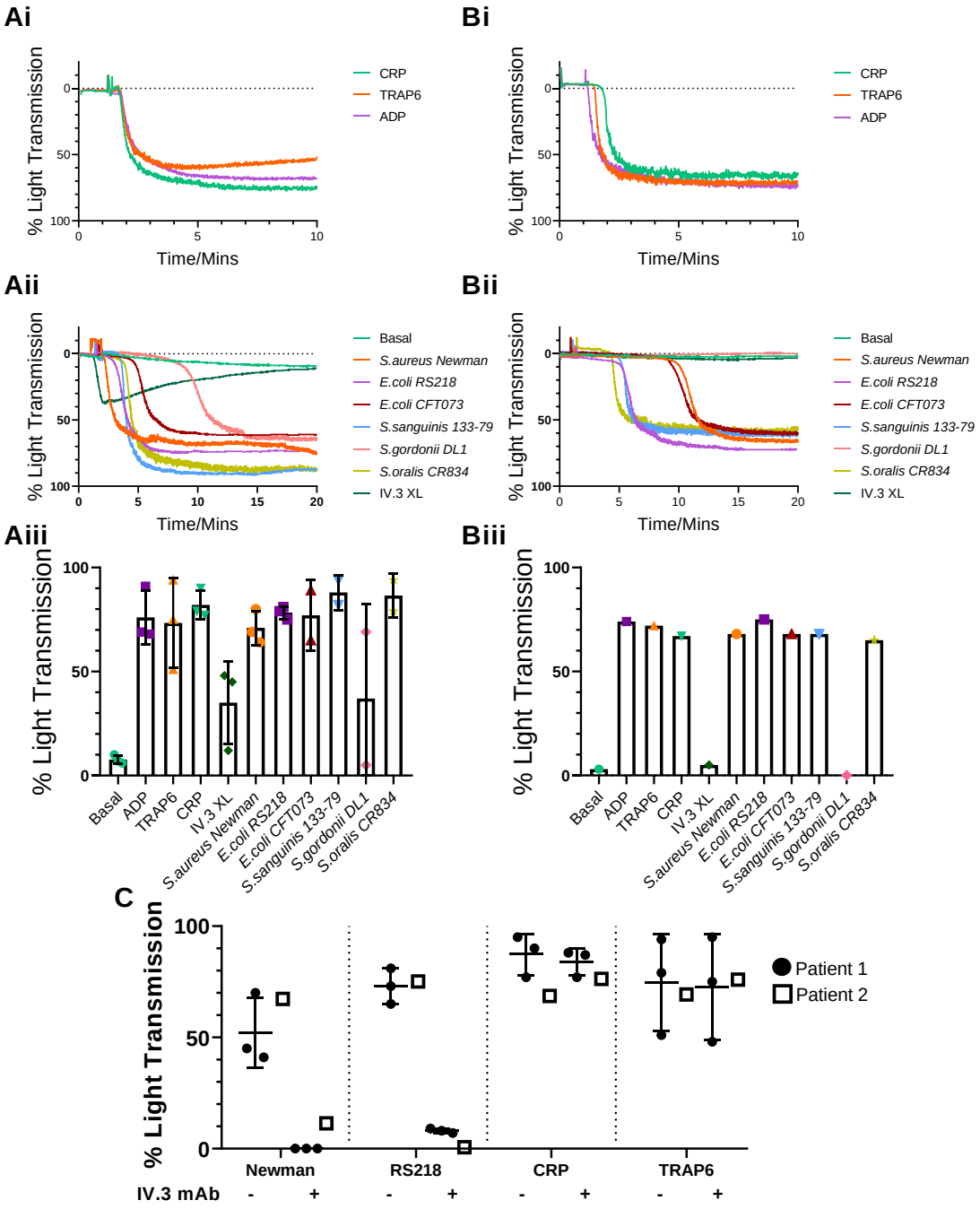


Figure 5.4. XLA patient platelets respond to bacteria despite the lack of functional Btk. XLA platelets from two XLA patients, (A) patient 1 and (B) patient 2, were analysed by LTA upon stimulation with (i) 3µg/ml CRP, 3µM of TRAP6 amide and 10µM ADP, or (ii) vehicle, 4µg/ml mAb IV.3 followed by 30µg/ml F(ab')$_2$ rabbit anti-mouse IgG, and assorted bacteria including *S. aureus* Newman, *E. coli* RS218, *E. coli* CFT073, *S. sanguinis 133-79, S. gordonii* DL1 or *S. oralis* CR834. (C) PRP was first incubated with vehicle or 20µg/ml IV.3 mAb for 10 minutes (to inhibit the FcγRIIa receptor) before the addition of either *S. aureus* Newman, *E. coli* RS218, 3µg/ml CRP or 3µM TRAP6 amide and measured by LTA. Graphs show mean ±SD.

5.2.5 Platelet Tec phosphorylation in response to bacteria is inhibited by ibrutinib

The results from the XLA patients demonstrate that platelet activation in response to FcγRIIa activation by bacteria can still occur despite the lack of functional Btk, which suggests an involvement of Tec. Other studies have suggested that ibrutinib exerts off-target effects on Tec in the GPVI pathway, potentially contributing to the bleeding tendency present in ibrutinib-treated CLL patients. Due to this, phospho-Tec ELISA experiments were carried out, first to determine if Tec was activated in response to bacteria in healthy controls and ibrutinib-untreated CLL platelets, then to investigate the potential effect of *in vitro* and *in vivo* ibrutinib on Tec phosphorylation.

Washed platelets from healthy controls were incubated with either ibrutinib or vehicle for 5 minutes before addition of either CRP, *S. aureus* Newman or *E. coli* RS218 followed by lysate collection. CRP was used as a control as Tec is known to be activated downstream of GPVI. Washed platelets from ibrutinib-untreated CLL patients were stimulated with CRP or *S. aureus* Newman, while ibrutinib-treated CLL platelet samples had just the addition of CRP.

As seen in Figure 5.5, bacteria stimulation of healthy control platelets induced platelet Tec phosphorylation. CRP stimulation increased levels of Tec phosphorylation more than bacteria stimulation, though this was not statistically significant. Ibrutinib-untreated CLL platelets were able to phosphorylate Tec to similar levels as the healthy controls in response to both bacteria and CRP. *In vitro* addition of ibrutinib to healthy controls inhibited phosphorylation of Tec in response to bacteria. Ibrutinib-treated CLL platelets had diminished Tec phosphorylation when activated with CRP.

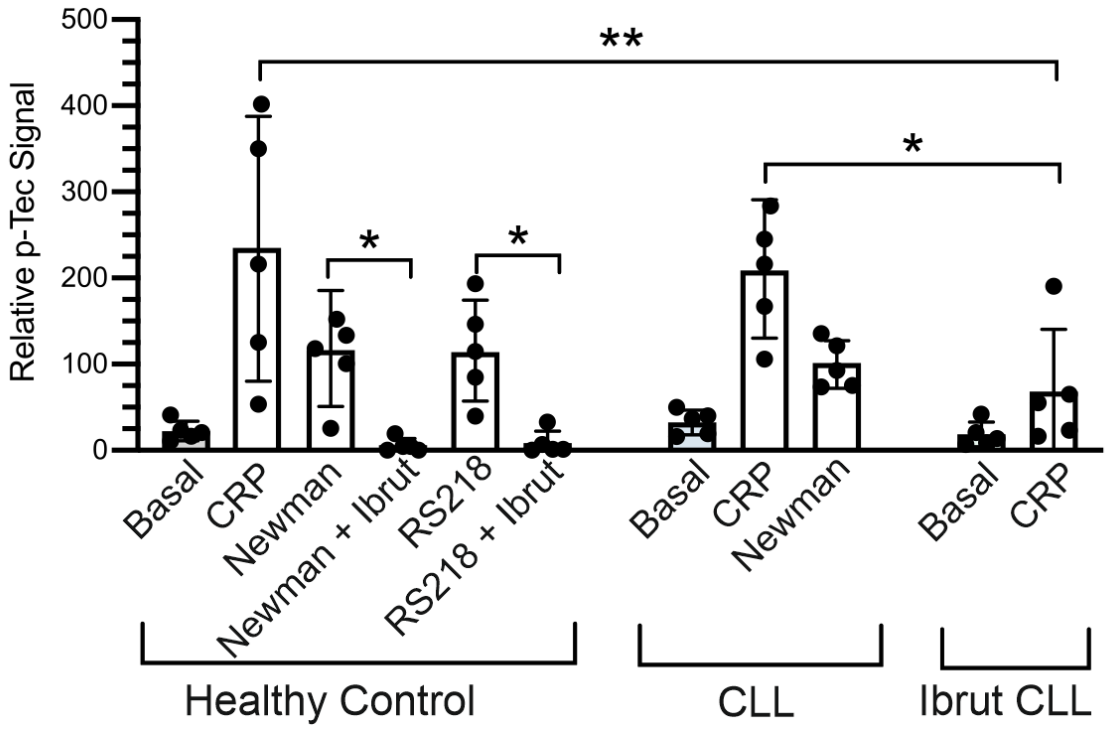


Figure 5.5. Platelet Tec is phosphorylated in response to platelet interaction with bacteria. Characterisation of platelet Tec phosphorylation in healthy donors, ibrutinib-untreated and ibrutinib-treated CLL patients. Washed platelets supplemented with fibrinogen (1mg/ml) and human IgGs (0.2mg/ml purified from healthy donors), were incubated with 5µM ibrutinib or vehicle for 5 minutes, before stimulation with either 3µg/ml CRP, *S. aureus* Newman or *E. coli* RS218. Relative phosphorylation of Tec was measured by phosphor-Tec ELISA. Data shows mean ±SD. *P<0.05, **P<0.01, ***P<0.001.

5.3 Discussion

The main findings from the studies above on the effects of ibrutinib on Btk signalling in platelets are:

- Platelet Btk and Tec are activated in response to bacteria.
- Btk is not essential for platelet activation in response to bacteria.
- Ibrutinib inhibits bacteria induced Btk and Tec activation in platelets.

A previous study (Oda *et al.*, 2000) has reported the phosphorylation of Btk and Tec downstream of collagen binding to GPVI as well as antibody-mediated FcγRIIa crosslinking. It is possible that Btk is activated when FcγRIIa is stimulated with bacteria. However, the previous study of platelet FcγRIIa signalling was evaluated upon crosslinking of the receptor with antibodies only. The results from this chapter demonstrate that Btk is activated downstream of FcγRIIa when platelets interact with bacteria.

The results presented here show that Btk is phosphorylated at a higher level when platelets are stimulated with CRP rather than with FcγRIIa agonists. For this, Btk phosphorylation in platelets stimulated with either CRP, IV.3 XL or bacteria was compared within donors for healthy controls and ibrutinib-untreated CLL patients. The copy numbers of both GPVI and FcγRIIa are similar in platelets (Neeves, 2021), as such the apparent weaker Btk signal seen for FcγRIIa agonists is not due to surface receptor expression. Nor is it probably due to cell membrane location of the receptors. When both FcγRIIa and GPVI are activated, the receptors localise to lipid rafts (Stylianos Bournazos *et al.*, 2009) (Locke *et al.*, 2002), therefore the localised Btk signal and global Btk signal within a platelet would be expected to be similar for both receptors. However, there could be differences between how the ligands interact with their corresponding receptors, as well as there is the possibility of different kinetics between the receptors.

Within this study, three minutes after the start of platelet aggregation was chosen as the point of lysis. However, it is possible that when FcγRIIa is activated, Btk is phosphorylated at greater levels at a different time point than when GPVI is activated.

For example, for the case of bacteria Btk could be more strongly activated during the lag time or the start of aggregation rather than when maximum aggregation is seen, and this will need future investigation. Interestingly, use of ibrutinib and acalabrutinib reduced Btk phosphorylation levels to below basal levels. This shows that there is possibly another way to sustain basal phosphorylation levels of Btk that does not depend on FcγRIIa and αIIbβ3 signalling but could still have a role in the initial steps of platelet interaction with bacteria.

TRAP6 amide mediated platelet stimulation results in phosphorylation of Btk, although in chapter 3 TRAP6-induced aggregation was not shown to be affected by either ibrutinib or acalabrutinib. The thrombin receptor, which is activated by TRAP6 amide, is G-protein-linked and is not directly linked to tyrosine kinases. The fact that Btk is phosphorylated in response to TRAP6 amide could be due to αIIbβ3 outside-in signalling because our experiments were performed in aggregation conditions (e.g., without blocking αIIbβ3 and secondary mediators for lysate collection). Src and Syk are known to be activated in thrombin stimulated platelets and are not required for platelet aggregation (Hughan *et al.*, 2007), and these proteins could be expected to phosphorylate Btk.

In this study, a lower concentration of ibrutinib was able to inhibit aggregation in response to IV.3 XL compared to CRP in healthy donor platelets, suggesting that the FcγRIIa signalling pathway has an increased ibrutinib sensitivity, and potentially increased Btk dependence, than the GPVI signalling pathway. A previous study using whole blood (Goldmann *et al*., 2020) used lower concentrations of ibrutinib to inhibit FcγRIIa-mediated platelet aggregation than those employed in this study. The difference seen in inhibitory concentrations of ibrutinib could be attributed to the experimental approaches used. While this study has involved LTA, Goldmann et al. utilised multiple electrode aggregometry which has been reported to have lower sensitivity than LTA (Al Ghaithi *et al.*, 2017), and did not perform signaling studies to detect the levels of Btk phosphorylation.

Ibrutinib's peak plasma concentration is around 0.3μM (Advani *et al.*, 2013). The data in this chapter shows that 2-5μM ibrutinib is required for complete inhibition of Btk,

while 0.2-0.5µM ibrutinib merely attenuates Btk phosphorylation. In this study, platelets derived from ibrutinib-treated CLL patients show complete inhibition of Btk phosphorylation, however the approximate 0.3µM peak plasma concentrations could not be expected to be enough to inhibit blood circulating platelets. The inhibition seen in these patients could possibly happen due to a previously proposed theory for acalabrutinib (Bye et al. 2017) in which acalabrutinib may accumulate in patients' platelets overtime due to daily intake. It is possible this could also happen in the case of ibrutinib. This accumulation would allow the drug to achieve higher concentrations than what is found in plasma. However, this hypothesis required evaluation.

There have been many studies investigating the effects of ibrutinib and acalabrutinib on the GPVI signalling in platelets (Bye *et al.*, 2015b) (Nicolson *et al.*, 2018b) (Kamel *et al.*, 2015) (Bye *et al.*, 2015a) and a more limited amount studying FcγRIIa signalling and iBtks (Goldmann *et al.*, 2020). The previous studies have proposed proteins which could be experiencing off-target effect of ibrutinib, including Src (Bye *et al.*, 2017) (Series *et al.*, 2019) (Nicolson *et al.*, 2018a), which is upstream of both Btk and Tec in ITAM receptor pathways. It has been shown that Src can be inhibited in washed platelets at concentrations of 0.5µM ibrutinib and higher (Levade *et al.*, 2014). This concentration of ibrutinib is used to inhibit aggregation in response to CRP in washed platelets, allowing for the possibility that the effect seen in these circumstances is due to Src inhibition. One study, though, concluded that while Src was inhibited by ibrutinib, this was not the cause of ibrutinib inhibition of GPVI signalling, which instead lies with Btk and Tec (Bye *et al.*, 2015a).

Ibrutinib may be affecting αIIbβ3 function as it is known to prevent αIIbβ3 outside-in signalling (Bye *et al.*, 2015a). In platelets, FcγRIIa signalling is dependent upon αIIbβ3 signalling for activation in response to bacterial agonists. Signalling through FcγRIIa results in the inside-out activation of αIIbβ3, allowing αIIbβ3 to bind to fibrinogen. Notably, some bacteria like *S. aureus* Newman bind αIIbβ3 in addition to FcγRIIa (Cox, Kerrigan and Watson, 2011). These first steps leading to FcγRIIa and αIIbβ3 activation result in ADP release from dense granules and *de novo* thromboxane production that initiates a positive feedback loop that is key for platelet activation to bacteria (Arman *et al.*, 2014). Therefore, inhibition of αIIbβ3 by ibrutinib could have an important

impact on platelet secretion and aggregation to bacteria. In contrast, stimulating FcγRIIa with crosslinked IV.3 mAb does not require αIIbβ3 to induce strong signalling and platelet secretion (Arman *et al.*, 2014), and as such, the effect of ibrutinib on platelet FcγRIIa function could be agonist dependent.

Inhibition of IV.3 XL mediated platelet activation by iBtks could be due to off-target effects on Tec. Additionally, Tec can substitute for Btk in the GPVI pathway (Atkinson, Ellmeier and Watson, 2003b) and is irreversibly inhibited by ibrutinib (Bye *et al.*, 2017) following GPVI activation. It is suggested that neither ibrutinib or acalabrutinib display selectivity for Btk over Tec (Chen *et al.*, 2018), though previously it was thought that acalabrutinib experienced a greater selectivity towards Btk over Tec than ibrutinib (Wu, Zhang and Liu, 2016) (Barf *et al.*, 2017). As such, it is possible that Tec is inhibited by iBtks affecting FcγRIIa activation.

Despite Tec being inhibited downstream of the GPVI signalling pathway using ibrutinib, another study (Goldmann *et al.*, 2019) looking into the effects of iBtks on FcγRIIa in HIT, claimed that Tec is not essential in the FcγRIIa signalling pathway. The study mentioned that although other studies have shown that increasing the concentrations of collagen can overcome the inhibition of Btk through the alternate use of Tec in the GPVI pathway, this was not the case for FcγRIIa receptor crosslinking with antibody. Moreover, the iBtk fenebrutinib, which has been shown to not inhibit Tec (Crawford *et al.*, 2018), was able to inhibit FcγRIIa-mediated platelet activation (Goldmann *et al.*, 2019).

Though this chapter focused on Btk phosphorylation at Y223 as a marker of Btk activation, it remained unknown if Btk is essential for platelet responses to bacteria through FcγRIIa. XLA patients are a best human model for platelets lacking functional Btk given the limitations of studying platelets, as a comparison can be made between these platelets and those from previous platelets in these studies, both from the healthy donors and CLL patients. While mice can be genetically modified to prevent expression of Btk, this only allows for the *Xid* phenotype, which is a lot milder than the phenotype seen for XLA patients potentially meaning a difference across species. Additionally, mice do not naturally express the FcγRIIa receptor, and while human

FcγRIIa transgenic mice are available (McKenzie *et al.*, 1999), mice also do not have immunoglobulin isotypes equivalent to humans, which may prevent a close to human response (seen in previous studies which isolated FcγRIIa-transgenic mouse platelets and supplemented with human IgGs and fibrinogen before incubation with bacteria which for most strains failed to result in platelet aggregation (Arman *et al.*, 2014)); while XLA patients receive a regular transfusion of human immunoglobulins, ensuring enough levels and of correct isotype.

XLA patients experience recurrent infections due to defects in B cell maturation and IgG production caused by the lack of functional Btk. However, Btk is also absent in other haematopoietic cells that normally express Btk, such as platelets. Nevertheless, XLA patients do not experience the bleeding phenotype that some ibrutinib-treated CLL patients display. This is thought to be due to the ability of Tec to substitute for Btk in XLA platelets as seen in mice knockout studies with platelets stimulated with collagen and CRP: in $Tec^{-/-}$ mice phosphorylation of PLCγ2 was unaltered, while $Btk^{-/-}$ mice were found to have dramatically decrease PLCγ2 phosphorylation. $Tec^{-/-}/Btk^{-/-}$ mice had a greater decrease in PLCγ2 phosphorylation than $Btk^{-/-}$ mice, highlighting the importance of Tec when Btk is absent (Atkinson, Ellmeier and Watson, 2003b). Tec could be blocked by ibrutinib in ibrutinib-treated CLL patients, suggested by data in this chapter. The current study demonstrates that XLA patients respond to CRP normally in contrast to ibrutinib-treated CLL patients. XLA platelets can respond normally to bacteria, again despite the lack of Btk, which would suggest that Tec could be substituting for Btk in the FcγRIIa pathway. However, platelet bacteria interactions can involve multiple molecular interactions, and other pathways could also be compensating for the lack of Btk. In contrast to XLA platelets, responses to bacteria are inhibited in platelets derived from ibrutinib-treated CLL patients, possibly due to the combined effect of ibrutinib on Btk and Tec, and perhaps other tyrosine kinases.

The phosphorylation of Btk Y223 allow for the full activation of Btk (Marcotte *et al.*, 2010). The equivalent activation site in Tec is Y206 (Nore *et al.*, 2003). The study of Tec in human platelets is technically more challenging than that of Btk due to a lack of specific phospho-Tec antibodies and a lack of an XLA-equivalent disease for Tec. A total phospho-Tec ELISA was performed in these studies. Though this ELISA gave valuable

novel information, the activation of Tec upon IV.3 XL could not be studied due artefactual errors caused by the presence of $F(ab')_2$ fragments and IV.3 mAb in the supernatants tested.

Tec ELISA data presented this chapter demonstrates that ibrutinib-treated CLL patients have diminished Tec phosphorylation responses to CRP in comparison to healthy controls and untreated-CLL. Additionally, the *in vitro* addition of ibrutinib to healthy control platelets inhibited Tec phosphorylation in response to bacteria. However, in a previous study (Goldmann *et al.*, 2019) using antibody-crosslinked FcγRIIa, the authors claimed that Tec is not of functional relevance to downstream FcγRIIa signalling. The authors concluded this as the reversible iBtk fenebrutinib, which does not inhibit Tec, completely inhibits FcγRIIa-mediated aggregation, though the study included no signalling assays. However, while the current study has shown that Tec is phosphorylated in response to bacteria, it does not show that Tec is essential to the signalling pathway.

Ibrutinib and acalabrutinib selectivity and IC_{50} for Btk and Tec is very similar in donors (Hopper *et al.*, 2020). However, the data presented herein does not definitively show that inhibition of platelet activation in response to bacteria by *in vivo* ibrutinib is solely mediated through Btk and Tec. More studies are needed to ascertain if other signalling proteins are also affected by ibrutinib downstream of FcγRIIa. However, previous studies (Nicolson *et al.*, 2018a) demonstrated that downstream of GPVI signalling, ibrutinib inhibition of Btk and Tec was irreversible, though inhibition of aggregation was reversible, suggesting that Btk and Tec may not be the only kinases inhibited by ibrutinib. Other studies specify Src as a possible target (Nicolson *et al.*, 2018a) (Bye *et al.*, 2017), with possible other targets which are currently unknown. Inhibition of other kinases by ibrutinib may also be seen in the FcγRIIa pathway.

These studies only encompass the iBtks ibrutinib and acalabrutinib. The findings presented in this chapter indicate that the iBtks have significant effects on FcγRIIa signalling. It has previously been reported that when time dependent factors have been accounted for, acalabrutinib has a similar selectivity for Btk over Tec as ibrutinib, with an IC_{50} of 1.09 and 1.32 respectively (Hopper *et al.*, 2020). This raises the

potential implications for other iBtks both in terms of selectivity for Btk and effect on the FcγRIIa pathway in platelets. As such, it is worth investigating other iBtks and their effects on platelets to establish their possible adverse effect profiles in both thrombotic and immune functions.

Chapter 6: Discussion

6.1. General discussion

CLL is the most common form of leukaemia in the Western world, and affected patients have a higher incidence of infection. CLL patients treated with the Btk inhibitor ibrutinib experience an even greater rate of infections (Ball *et al.*, 2020), as well as bleeding complications (Wang *et al.*, 2020). Platelets are primarily known for their role in haemostasis and thrombosis and were quickly linked to the bleeding phenotype in ibrutinib-treated patients (Kamel *et al.*, 2015) (Levade *et al.*, 2014). Platelets are less well known for their immunological role. Platelets express immune receptors which mediate platelet-bacteria interactions causing platelet activation and aggregation, granule secretion and activation of other immune cells. One main immune receptor expressed by platelets is FcγRIIa, which binds IgG opsonised bacteria, and whose signalling pathway involves Btk, the target of ibrutinib.

This thesis demonstrates that ibrutinib, and the second-generation Btk inhibitor acalabrutinib, inhibit FcγRIIa mediated platelet immune responses. This was accomplished by measuring a range of platelet activation read-outs in healthy controls, untreated CLL and ibrutinib-treated CLL patients after stimulation by crosslinking the FcγRIIa receptor via IV.3 mAb, or by the bacteria *S. aureus* Newman and *E. coli* RS218. The thesis also explores the possible effects of iBtks on other signalling proteins in the FcγRIIa signalling pathway.

The data presented in this thesis demonstrated that:

- iBtks, ibrutinib and acalabrutinib, impair FcγRIIa-mediated platelet activation in healthy donor platelets in response to FcγRIIa cross-linking as well as to stimulation by bacteria.
- CLL-derived platelets respond to bacteria via the FcγRIIa pathway and ibrutinib treatment impairs such responses.
- Ibrutinib treatment impairs platelet scavenging of bacteria in CLL patients.

- Ibrutinib treatment does not affect cell surface expression of FcγRIIa on CLL platelets.
- Btk is activated through FcγRIIa signalling in response to bacteria in healthy donors and ibrutinib-untreated CLL patients, and this activation is inhibited in ibrutinib-treated CLL patients.
- *In vitro* addition of ibrutinib and acalabrutinib inhibits Btk activation in healthy donor platelets.
- Platelet-expressed Tec is activated in response to bacteria and this activation is inhibited via *in vitro* addition of ibrutinib to healthy controls. Tec phosphorylation is impaired in ibrutinib-treated CLL platelets.

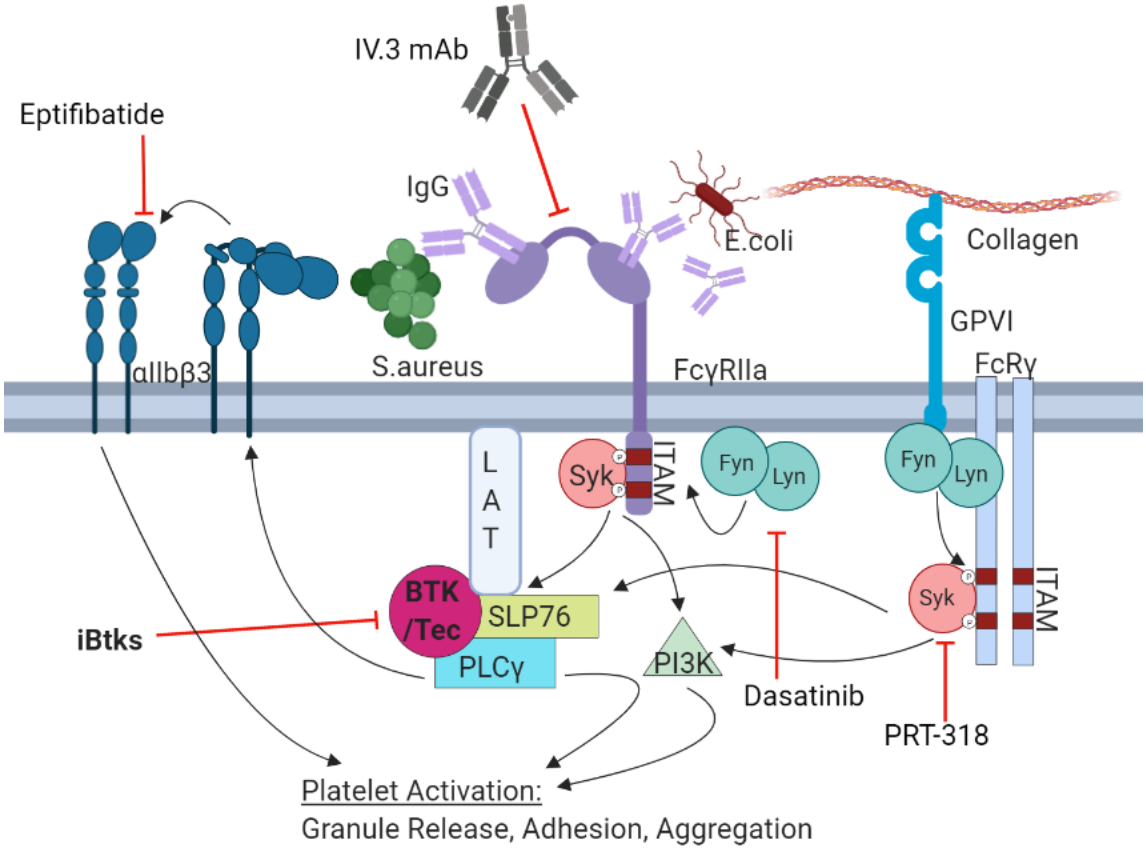


Figure 6.1. Platelet signalling through FcγRIIa, GPVI and αIIbβ3. Figure showing kinase activity after signalling through FcγRIIa, GPVI and αIIbβ3 including inhibitors used within this study.

Infections are a leading cause of death in CLL (Lee *et al.*, 1987), with 30-50% mortality due to infection and 67% of such infections being bacterial (Molica, 1994). This increased risk of infections and changes in cell-mediated immunity are due to several

factors, including prior treatment, disease stage, age, comorbidities and patient fitness (Balducci and Dolan, 2015). CLL patients typically have a normal total T-cell count, but reduced numbers of T-helper cells. In contrast there are increased number of T suppressor cells, and natural killer cell deficiencies (Platsoucas *et al.*, 1982). The patients can have chronic stimulation of neutrophils inducing tolerance, and impaired differentiation of dendritic cells (Arruga *et al.*, 2020). Advanced clinical stage of CLL or poor therapy response is another risk factor for infection (Morrison, 2010). Advanced clinical stage of CLL is associated with more frequent and severe infections with the rate of infection being 33% for Stage A and 82% for Stage C patients (Itala *et al.*, 1992).

In advanced stage CLL there are other haematological and immunological changes, such as thrombocytopenia, lymphocytosis (Rai *et al.*, 1975) and hypogammaglobulinaemia. The latter is present in around 10-100% of CLL patients (Itala *et al.*, 1992) and affects all Ig classes (Forconi and Moss, 2015). A decrease in IgG is associated with increased morbidity and mortality by infections (Rozman, Montserrat and Viñolas, 1988). There is a decrease in IgG2 and IgG3 subclasses of Ig in CLL (Rossi *et al.*, 2009) (Rozman, Montserrat and Viñolas, 1988) (Jurlander, Geisler and Hansen, 1994). The IgG subclasses have preferential binding affinities for FcγRIIa in the order of IgG3, IgG1, IgG2 and lastly IgG4. IgG subclasses 1 and 3 are seen to have a slight increase in the elderly, but it is through IgG2 by which most bacterial interactions occur and hence result in cell activation.

Taking these factors all together, a patient with a 5-year history of CLL has a risk of 26% of severe infection. If this patient also has hypogammaglobulinemia then this risk increases to 57%. If this patient also has Stage C CLL, then the risk of infection from these accumulated factors is 68% (Molica, 1994).

Ibrutinib treatment has been linked to an increased risk of infection (Andersen *et al.*, 2019) (Varughese *et al.*, 2018) (Chaul Barbosa, DeAngelis and Grommes, 2017) (Burger *et al.*, 2015) (Byrd *et al.*, 2013) (Chanan-Khan *et al.*, 2016) (Byrd *et al.*, 2014) (Byrd *et al.*, 2015). Morbidity related to infections in ibrutinib-treated patients decreases over time possibly because ibrutinib has the potential to partially reconstruct the humoral

immune response by increasing the levels of immunoglobulins, on top of levels of the normal non-malignant B-cells (Sun *et al.*, 2015).

Staphylococcal interactions with healthy platelets are well described (Hamzeh-Cognasse *et al.*, 2015), and hence why *S. aureus* Newman was chosen in this study. *S. aureus* expresses several proteins which mediate platelet aggregation (O'Brien *et al.*, 2002). ClfA is one of these *S. aureus*-expressed proteins, as well as fibronectin-binding proteins, both of which bind to the same area of fibrinogen thereby activating αIIbβ3 and engaging FcγRIIa (Fitzgerald, Foster and Cox, 2006). The expression of ClfA and fibronectin-binding proteins is dependent on the growth phase of *S. aureus.* In stationary phase, ClfA is used for platelet aggregation (Loughman *et al.*, 2005), whilst in exponential growth fibronectin-binding proteins are used (Fitzgerald *et al.*, 2006). In this thesis, bacteria were grown in an overnight culture and as such would be in stationary phase of growth and expressing ClfA. However, infections in the body would possibly be at exponential growth phase, and as such fibronectin-binding proteins would be expressed. This study did not investigate exponentially growing bacteria and how this would affect platelet aggregation and inhibition by ibrutinib.

Similar to *S. aureus*, *E. coli* is able to utilise the FcγRIIa receptor to cause platelet activation (Moriarty *et al.*, 2016) (Watson *et al.*, 2016). In contrast to *S. aureus*, little is known about the components of *E. coli* which interact with IgGs and FcγRIIa. Moreover, *E. coli* is known to interact with platelets in numerous ways which do not require the FcγRIIa receptor. *E. coli* can activate platelets via TLR4 (Matus *et al.*, 2017), with inhibition of the receptor blocking the increase of P-selectin and hence α-granule release. Platelet TLR4 activation through Gram-negative bacteria LPS results in caspase-11 transcription and signalling through the NLRP3 inflammasome. LPS activation of platelet TLR4 can result in the release of IL-1β in microparticles. IL-1β in microparticles can activate endothelial cell IL-1R resulting in vascular cell adhesion molecule 1 expression leading to the adhesion to such cells as lymphocytes and monocytes, resulting in local thrombosis and inflammation. Btk is found downstream of TLR4 and the inflammasome in platelets (Vogel *et al.*, 2018) (Vogel and Thein, 2018). Interestingly, crosstalk has been found between FcγRIIa and TLR4 in neutrophils. FcγRIIa levels on the surface of neutrophils are increased with the presence of LPS,

resulting in a closer distance between the two receptors, and when TLR4 is activated via IL-8, there was found to be FcγRIIa engagement, which mechanistically requires Btk (Krupa *et al.*, 2013). It is possible that this too could occur in platelets. The signalling through TLR4 could be inhibited by the iBtks too, and as such would be worthy of further investigation.

Activation of TLR4 may be strain dependent as previous experiments were performed with EHEC O111 (Matus *et al.*, 2017), while other *E. coli* strains, such as CFT073 and RS218, have been shown to activate platelets through FcγRIIa, without an apparent involvement of TLR4 (Watson *et al.*, 2016). *E. coli* can interact with TLR2 and the scavenging receptor GPIV (Lenehan *et al.*, 2016). However, *E. coli* RS218 does not seem to utilise TLR4, so it seems unlikely that this is a way in which aggregation could be initiated (Watson *et al.*, 2016). These results suggest that the FcγRIIa pathway is the predominant mediator of signalling responses to *E. coli*.

Within the vasculature, platelets scan, migrate and gather bacteria in a process called scavenging. This thesis shows that ibrutinib-treated CLL patients have impaired bacteria scavenging. It has previously been shown that fibrinogen scavenging is dependent upon αIIbβ3 (Gaertner *et al.*, 2017), and that platelets scavenge fibrin bound *S. aureus* and *E. coli* by a mechanism dependent upon actomyosin to pull bacteria to the platelet surface (Gaertner *et al.*, 2017). In another study, antibody opsonisation and platelet spreading over the opsonised *E. coli* occurred via FcγRIIa after PF4 binding to *E. coli* (Palankar *et al.*, 2018). Though this thesis does not address if FcγRIIa has a role in scavenging, it has previously been found that the inhibition of FcγRIIa with IV.3 mAb significantly decreases platelet scavenging of *S. aureus* Newman (Chacko, 2020) (Nicolson, 2018). Btk is downstream of both FcγRIIa and αIIbβ3, and upstream of PLCγ2. Activation of PLCγ2 results in inside-out activation of αIIbβ3 as well as the activation of Rho-GEFs which are required for cytoskeletal rearrangements. It has been seen in T cells that Btk and Tec have a role in actin polymerisation (Finkelstein and Schwartzberg, 2004) (Sharma, Orlowski and Song, 2009), as well as Btk having a role in Rac-dependent rearrangement of actin filaments in mast cells (Yao *et al.*, 1999) (Kuehn *et al.*, 2010). It is possible that the two kinases also play a role in actin skeletal rearrangements in platelets, and this could be another way in which use of

ibrutinib impairs platelet spreading and scavenging. Btk inhibition by ibrutinib therefore could reduce platelet scavenging and inhibit this immune process.

Polymorphonuclear neutrophils interact with platelets which have scavenged and accumulated bacteria, before phagocytosis and formation of NETs (Gaertner *et al.*, 2017). Platelets play a significant role in NET formation in many diseases, with activation of TLR9 (Matsumoto *et al.*, 2021) and TLR4 (Clark *et al.*, 2007) and FcγRIIa (Perdomo *et al.*, 2018) (Perdomo *et al.*, 2019) being the key platelet receptors in this process. Btk is downstream of TLR2 and 9 in other immune cells (Rip *et al.*, 2019) (Taneichi *et al.*, 2008). Therefore, ibrutinib may prevent or reduce NET formation due to the inhibition of Btk on platelets in addition to other immune cells and hence increase infection risk. As such, further investigation into the impact of ibrutinib on different immune cells including platelets in the context of infection in CLL is needed.

Aggregation of platelets and other cell-cell interactions are key elements in immunothrombosis, a process required for the induction of coagulation and thrombus formation in infection. The thrombi thus formed allow the capture and killing of pathogens. One such example of immunothrombosis is the fibrin clot mediated capture of *Streptococcus* and the subsequent induction of bacterial killing (Påhlman *et al.*, 2013). Mice had increased bacterial burden and death when lacking TF, a key protein in the initiation of thrombin formation and hence thrombi development (Luo *et al.*, 2011). Moreover, these thrombi can contain many different immune cells which platelets can activate through secretion of chemokines, such as CXC 1, 4, 5 and 7, which are then able to perform their immune functions. From the studies in this thesis, platelet aggregation and secretion are impaired with the use of the iBtks ibrutinib and acalabrutinib. Although no bacterial growth assays were performed in this study, based on the literature (Palankar *et al.*, 2018) (Riaz *et al.*, 2012) it can be hypothesised that the inhibition of Btk would reduce bacterial killing by platelets, and future studies should be conducted to investigate this. In certain diseases such as infective endocarditis, platelet-bacteria aggregates can be detrimental (Herzberg, 1996) and as such the use of ibrutinib could be beneficial in inhibiting the aggregate formation which further studies should investigate.

Due to platelets no longer being able to undergo FcγRIIa-mediated aggregation response to bacteria when inhibited by iBtks, thrombus formation would be expected to occur over a longer duration, no longer being a rapid response. Instead, it could be possible that the platelets would rely on other immune cells in the presence of iBtks, such as monocytes which have been shown to secrete TF from microvesicles, to stimulate platelet aggregation to a pathogen to begin the process of immunothrombosis. Additionally, other immune cells are inhibited by iBtk. Examples include macrophages and T cells, in which secretion was impaired (Colado *et al.*, 2018). Neutrophils exposed to iBtk treatment have been found to have reduced release of neutrophil elastase (Prezzo *et al.*, 2019), a pro-coagulation protein for platelets, and as such this could slow the formation of thrombi, and hence reduce the capturing and killing abilities associated with immunothrombosis.

In addition to impaired pro-coagulation factor release from platelets associated with iBtks, the secretion of substances involved in bacterial killing will also be inhibited. Thrombocidins are one such released protein, which are bactericidal against microorganisms such as *S. aureus*, *E. coli*, *Bacillus subtilis* and *Lactococcus lactis* (Krijgsveld *et al.*, 2000). Releasate from platelets has been found to decrease the number of bacteria (Dankert *et al.*, 1995), while PF4 released from α-granules can enter red blood cells and lyse vacuoles in which *Plasmodium* parasites are located in cases of malaria, killing the intracellular parasite (McMorran *et al.*, 2014). PF4 released from α-granules can promote platelet-leukocyte interactions. Neutrophil activation can occur via tetrameric PF4 binding to proteoglycan chondroitin sulphate which results in secondary granule exocytosis of neutrophils as well as increased adherence to endothelial cells (Petersen *et al.*, 1998). Platelets also release fibrinogen which binds to neutrophil-associated CD18 (Zhang, Zhan and Shin, 2013), possibly increasing adhesion of neutrophils to endothelial cells and platelets. Overall, the inhibition of aggregation and secretion in platelets can prevent the release of antimicrobial substances from platelets, as well as impair clot formation and hence bacterial capturing, and platelet-leukocyte interactions, which could contribute to the increased infection risk seen in patients with B cell malignancies taking ibrutinib.

Currently there are many Btk inhibitors both approved and in trials. Acalabrutinib and zanubrutinib are the most studied iBtks after ibrutinib and are meant to have greater selectivity for Btk as well as reduced adverse effects in patients (Byrd et al., 2016) (Wu, Zhang and Liu, 2016) (Tam et al., 2020). Ibrutinib has a reported risk of infection between 33-81% as monotherapy (Byrd *et al.*, 2013) (O'Brien *et al.*, 2014) (Maruyama *et al.*, 2016) (Wang *et al.*, 2013) (Tobinai *et al.*, 2016). In one study, 14% of patients receiving acalabrutinib as monotherapy experienced a significant infection compared with 21% of patients receiving acalabrutinib in combination with the anti-CD20 monoclonal antibody obinutuzumab (Sharman *et al.*, 2020). This demonstrates that while infection rates seem to lower with acalabrutinib treatment when compared to ibrutinib, the risk remains significant. Based on the data presented in chapter 3 of this thesis in which acalabrutinib was able to strongly inhibit the platelet responses of aggregation and granule secretion in response to bacteria, further studies should be performed assessing the *in vivo* effects of this drug on platelets.

It is worth noting that the use of dasatinib, PRT-318, ibrutinib and acalabrutinib on PRP was able to reduce the levels of Btk phosphorylation to below the levels seen in basal conditions. This was not true for the receptor inhibitors of eptifibatide and IV.3 mAb. An inverse agonist is described as a ligand which interacts with the same signal-transduction as agonist but exerts the completely opposite effect (Harkema, Nikula and Haschek, 2015). Ibrutinib and acalabrutinib bind to C481 in the kinase domain of Btk preventing the phosphorylation of Y551 and the autophosphorylation of Y223, which could be said to mimic an inverse agonist as the levels of phosphorylation drop below basal, which is the opposite of when an agonist is used in the signalling pathway in which phosphorylation levels increase. The same can be said of both PRT-318 and dasatinib.

The results of this thesis show that both ibrutinib and acalabrutinib have inhibitory effects upon platelet mediated immune responses. Future work should focus on other iBtk and how they affect immune responses. Infection remains a leading cause of mortality for CLL patients. Therefore, a deeper understanding of how iBtks influence immune responses in CLL-patients could help in the identification of patients at particular risk of major infection whilst treated with these drugs.

6.2. Future Work

The work carried out in this thesis has provided many interesting avenues to explore further.

- Platelets are known to activate other immune cells via a variety of cellular processes. This thesis demonstrates that iBtks affect platelet granule release which in turn would be expected to limit the activation of other immune cells through platelets. One example of platelet interaction with other immune cells is found in NETs, where platelet interaction with a neutrophil promotes the release of neutrophil DNA as well as granular and nuclear proteins, with subsequent entrapment and killing of pathogens (McDonald *et al.*, 2012). Platelets have been found to form circulating complexes with neutrophils in many infections and sepsis (Kirschenbaum *et al.*, 2002), which frequently occur in CLL, and, in animal models, these complexes aid in the recruitment of neutrophils to inflamed tissue. The role of ibrutinib on platelet and neutrophil interactions (Zhou *et al.*, 2020) and NET generation should be explored.

- Previous studies have shown that there is Btk-dependent interplay between TLR4 and FcγRIIa in neutrophils (Krupa *et al.*, 2013). Both TLR4 and FcγRIIa are found in platelets, so it would be interesting to explore if similar interactions can be observed, and if so, if ibrutinib causes an inhibition.

- In this thesis it is demonstrated that XLA patient platelets respond to bacteria despite the lack of functional Btk. Only two XLA patients were used in this study due to a lack of local availability of patients with this condition. The assays in this thesis should be repeated with a larger sample size to better characterise platelet immune responses in XLA. Also, this thesis suggests that Tec is substituting for Btk downstream of FcγRIIa in XLA patients. As such, the characterisation of the role of Tec in platelet immune responses should be studied.

- When this study was initiated, and for most of its duration, there was no access to CLL patients taking acalabrutinib. The result of this was all experiments with acalabrutinib were carried out *in vitro*. Acalabrutinib is known to be more specific to Btk than ibrutinib and patients taking acalabrutinib have a lesser risk of bleeding. As such it would be interesting to repeat the assays performed with ibrutinib-treated CLL platelets in this thesis with acalabrutinib-treated CLL platelets.

- The most common bacteria seen in CLL patients are *Streptococcus pneumoniae* and *Haemophilus influenzae*, while fungi are also common. Due to this, it would be interesting to further study CLL platelet responses to these bacteria and fungi, and to see the effect of iBtks on such responses.

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