PATHOGENIC ANTIBODIES OF HEPARIN-INDUCED THROMBOCYTOPENIA

DESCRIBING THE EPITOPES OF PATHOGENIC ANTIBODIES IN HEPARIN-

INDUCED THROMBOCYTOPENIA

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Lay Abstract

At least 30% of patients admitted into the hospital will be exposed to the anticoagulant, heparin. 1-3% of these patients develop heparin-induced thrombocytopenia (HIT): an adverse drug reaction. HIT is a major cause of morbidity and mortality in patients receiving heparin if not diagnosed and treated in a timely manner. HIT occurs when patients form antibodies against the platelet protein, platelet factor 4, in complex with heparin leading to an immune response. However, most heparin-exposed patients produce these antibodies but do not have HIT. Current rapid and available diagnostics tools cannot distinguish between antibodies that can or cannot cause the disease. To improve HIT diagnosis, we will identify the molecular differences between the antibodies that cause HIT and those that do not. From this, we can develop a new diagnostic assay that will be able to dictate whether the antibodies found in patients are specific for HIT.

Abstract

Heparin is an anticoagulant widely administered to patients undergoing major orthopedic or cardiac surgery. Though heparin is effective at preventing thrombosis, it is paradoxically associated with the development of heparin-induced thrombocytopenia (HIT). HIT is strongly associated with thrombotic complications and is an adverse drug reaction that occurs when heparin binds to the self-protein, platelet factor 4 (PF4) and forms immunogenic multimolecular complexes. As a result, anti-PF4/heparin antibodies are formed, which bind to these complexes, and can cross-linking Fc receptors on platelets and monocytes causing intense platelet activation, thrombocytopenia, and

thrombosis. Patients who receive heparin frequently form antibodies against these PF4/heparin complexes; however, most of these antibodies do not cause HIT. Over-diagnosis of HIT is common due to the detection of clinically insignificant non-pathogenic anti-PF4/heparin antibodies. Current enzyme immunoassays (EIAs) cannot distinguish between pathogenic and non-pathogenic anti-PF4/heparin antibodies and will give a false positive result in the presence of the clinically insignificant non-pathogenic anti-PF4/heparin antibodies. Further functional testing is required to identify samples containing the pathogenic anti-PF4/heparin antibodies that will lead to HIT; however, these tests are not readily available in most centres, and delay timely diagnosis. There is little known about the differences between pathogenic and non-pathogenic HIT antibodies. The identification of antigenic determinations of pathogenic HIT antibodies binding to PF4 from this project will have direct implications for patient care. We will be able to accurately and rapidly identify "true" HIT patients from learning more about the pathogenic HIT antibody epitope.

Acknowledgements

Table of Contents

Lists of Figures

List of Tables

List of all Abbreviations and Symbols

BCA	Bicinchoninic acid
BLI	Biolayer interferometry
BS^3	Bis (sulfosuccinimidyl) suberate
BSA	Bovine serum albumin
CHAPS	3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate
CPB	Cardiopulmonary bypass
DNA	Deoxyribonucleic acid
DOC	Deoxycholic acid
DVT	Deep vein thrombosis
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
GAGs	Glycosaminoglycans
HIPA	Heparin-induced platelet activation assay
HIT	Heparin-induced thrombocytopenia
IgG	Immunoglobulin G
IPTG	Isopropyl -D-1-thiogalactopyranoside
kDa	Kilodaltons
LB	Luria-Bertani
LC-MS	Liquid chromatography-mass spectrometry
LDAO	Lauryldimethylamine-oxide
LMWH	Low molecular weight heparin
mAb	Monoclonal antibody
MALDI-TOF	Matrix assisted laser desorption ionization time-of-flight
MFI	Mean fluorescence intensity
MW	Molecular weight
NMR	Nuclear magnetic resonance
OD600	Optical density (600 nm)
PBD	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PEA	P-selectin expression assay
PF4	Platelet factor 4
PNPP	p-nitrophenylphosphate
rhPF4	Recombinant human platelet factor 4
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SRA	^{14}C-serotonin release assay
UFH	Unfractionated heparin
VWF	von Willebrand factor

Declaration of Academic Achievements

The experiments outlined in this thesis were designed, conducted, and interpreted by myself and my supervisors, Dr. Ishac Nazy, Dr. Donald M. Arnold, and Dr. John G. Kelton, unless specified otherwise in the author preface. Platelet preparations of PF4 and patient sample collection was done by Jane C. Moore (Chapter 2). Human platelet preparation and PF4-SRAs were performed by Hina Bhakta and James W. Smith (Chapter 3 and 5). BLI experiments of confirmed and suspected HIT patient serum samples were performed by Rumi Clare and Marina Ivanova (Chapter 4).

Chapter 1

Introduction

1.1 Platelets

Platelets are nonnuclear derivatives of megakaryocytes and smallest and most abundant corpuscular component of blood (Ogawa, 1993, Ribatti and Crivellato, 2007). Their shape is usually discoid but upon activation, they undergo a shape change to a globular form with pseudopodia. Platelets circulate in blood vessels and do not ordinarily interact with other platelets or cell types (Tangelder et al., 1985). Platelets only last 8-10 days in circulation with 100 billion new platelets produced daily from bone marrow megakaryocytes in order to maintain normal platelet counts (Grozovsky et al., 2015). Platelets are coated on their surface with glycoproteins, which prevent adherence to endothelial cells. However, once activated, the platelets release their intracellular granules that mediate further platelet activations.

Found within platelet granules are many molecules required for hemostatic function. In the hemostatic response to injury, platelets are activated by collagen and

thrombin (Tangelder et al., 1985). Collagen fibrils within the vessel wall become exposed to the circulation when the endothelial cell monolayer is breached, forming a complex with von Willebrand factor (VWF) (Pietu et al., 1987). Platelets tumbling on the periphery of the rapidly moving bloodstream are captured when glycoprotein GP Ibα on the platelet surface binds to the VWF, establishing contacts that slow the forward progress of the platelet long enough for platelet activation to occur (Kroll et al., 1991). Drivers for platelet activation include the signaling events that occur downstream of receptors for collagen (GP VI and GP Ibα), thrombin (PAR1 and PAR4), adenosine diphosphate (ADP; $P2Y_1$ and $P2Y_{12}$), and thromboxane A_2 (TxA_2; TP) (Puri and Colman, 1997, Coughlin, 2005, Paul et al., 1999).

Primary hemostasis leads to the formation of a platelet plug. When wall injury occurs, vascular constriction is the first response of the body to immediately diminish the loss of blood. By reducing the vessel lumen, the flow rate decreases, and consequently, the blood loss reduces. Activated platelets provide an efficient catalytic surface for the assembly of the enzyme complexes of the blood coagulation system. When endothelial cells are damaged, platelets start to adhere (Cines et al., 1998). At the same time, exposition of collagen and binding of VWF to exposed collagen activate the platelets. Activated platelets change their shape completely: they swell and start to form long pseudopods that stretch out from their surface. Platelet adhesion increases that allows adhesion of platelets to the damaged endothelium and to other platelets (Varga-Szabo et al., 2008, Jackson, 2007). The activated platelets release large quantities of granules,

which act on surrounding platelets to activate them as well. The final platelet plug is comprised of inactivated and activated platelets (Hanasaki and Arita, 1988, Kahn et al., 1998, Vu et al., 1991). If the injury is not small, the platelet plug alone cannot close it completely. Secondary hemostasis will lead to the formation of a blood clot. A blood clot consists of a fibrin matrix, in which red and white blood cells, platelets, and plasma are entrapped (Furie, 2009).

1.2 Heparin and its anticoagulant properties

Heparin is a naturally occurring anticoagulant used for thromboprophylaxis or treatment in certain clinical situations, such as cardiovascular surgery, acute coronary syndromes, and dialysis (High, 1988, Hirsh et al., 2001). Heparin was accidentally discovered by McClean, a second-year medical student at the Johns Hopkins Medical School in 1916. Further studies of heparin were performed by his eminent supervisor, Dr. Howell, who named it heparin after its extraction from animal hepatic tissues (Howell, 1928). Despite being discovered in 1916, it was not used immediately in clinical and practical settings until twenty years after its discovery by Best and Murray (Murray et al., 1936, Best, 1938). By the 1950s, heparin was established as an important therapeutic agent in the treatment of venous and arterial thrombosis.

Heparin mediates its anticoagulant effect through inactivation of thrombin and factor Xa by binding and enhancing activation of antithrombin III through a high-affinity pentasaccharide pattern (Barrowcliffe et al., 1989). Having an immediate onset of action,

relatively short half-life, ability to be reversed, low cost, and be easily monitored in the laboratory has resulted in the broad use of heparin over other anticoagulant alternatives (Kelton and Warkentin, 2008). The heparin polymer ranges in molecular weight from 1,800 to 30,000 Daltons. Heparin sizes can be classified as unfractionated heparin (UFH), which includes the most variety of lengths, low molecular weight heparin (LMWH), and the synthetic fondaparinux, the smallest subset of heparin (Weitz and Weitz, 2010). All preparations of heparin have been used as treatment, with UFH being the most frequently administered (Warkentin et al., 1995). In some cases, however, heparin can result in a serious drug reaction that paradoxically leads to an increase in thrombosis (Warkentin and Sheppard, 2006, Martel et al., 2005).

1.3 Heparin-induced thrombocytopenia (HIT)

The ability of heparin to cause thrombocytopenia was first reported by several investigators in the 1940s in dogs and mice (Copley and Robb, 1942, Quick et al., 1948). In 1962, this same phenomenon was described in humans, but early reports did not view this to be a clinically significant problem (Gollub and Ulin, 1962, Davey and Lander, 1968). The link between thrombocytopenia and thrombosis was first recognized by Rhodes and his colleagues in 1973 (Rhodes et al., 1973). They reported that two patients had developed thrombocytopenia during heparin therapy that resolved when heparin was ceased. Additionally, thrombocytopenia promptly reoccurred when challenged again with heparin. They further demonstrated a heparin-dependent platelet aggregating factor in the patients' sera that could be found in the IgG fraction. The 1980s saw the concept of two

clinical types of HIT arise (Chong et al., 1982). One group of patients had mild thrombocytopenia with no detectable heparin-dependent IgG platelet aggregating factor in their sera. The second group of patients had severe, delayed-onset thrombocytopenia and frequently suffered thromboembolic complications. A heparin-dependent IgG platelet aggregating factor was found in the second group of patients. During the 1990s, the main characteristics of the pathogenesis and clinical presentation of HIT were revealed.

Heparin-induced thrombocytopenia (HIT) is a prothrombotic and potentially life-threatening disorder that develops in 1-5% of patients exposed to intravenous heparin (Kelton, 2002, Kelton and Warkentin, 2008). HIT characteristically occurs between 4 and 10 days after the initial heparin administration and can lead to patients being permanently disabled by amputation, stroke, or death in 20-30% of cases (Ahmed et al., 2007). HIT is mediated by platelet activating antibodies against their target antigen. The antigen was first identified by Amiral *et al.* (1992) to be a complex of heparin and the platelet protein, platelet factor 4 (PF4, also known as CXCL4).

Platelets and megakaryocytes carry in their α-granules PF4, a small cationic chemokine with multiple functions in the body (Mayo and Chen, 1989). When platelets are activated, PF4 is released and binds to glycosaminoglycans (GAGs) on the platelet surface (Figure 1.1). Heparin and other GAGs bind PF4 with high affinity due to the attraction of their opposite charges (Krauel et al., 2011). Upon binding, PF4 undergoes a conformational change and exposes neoepitopes that act as an immunogen (Kelton and

Warkentin, 2008, Kreimann et al., 2014) when the positive charge of PF4 is neutralized by polyanions. In this interaction, PF4 molecules can come into close proximity with one another, creating linear, ridge-like protein complexes (Greinacher et al., 2006). This reduces the distance between PF4 molecules to 3-5 nm. These clusters are recognized by anti-PF4/heparin antibodies, triggering an immune response (Figure 1.1). Other polysaccharides, negatively charged compounds, and hyper-sulfated chondroitin sulfate can also bind PF4 and induce the same conformational change (Brandt et al., 2014).
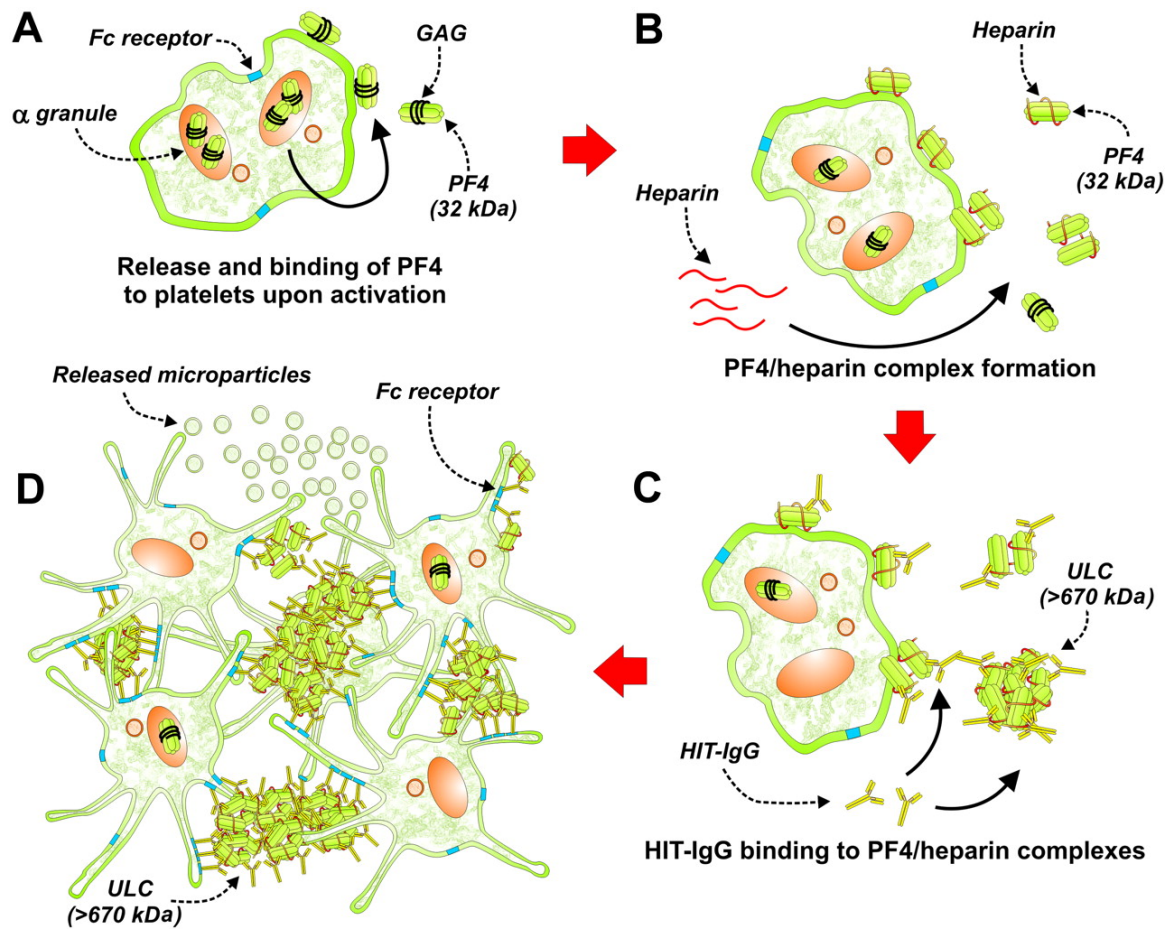
Figure 1.1: A schematic representation of the interaction anti-PF4/heparin IgG with platelets. A) Platelet factor 4 (PF4) is released from platelet α-granules after platelet

activation. The PF4 binds to the platelet surface through glycosaminoglycans (GAGs) that are associated with tetramers of PF4. B) Heparin binding to the PF4. C) Anti-PF4/heparin IgG antibodies binding to epitopes on the PF4/heparin complex. D) Ultra-large complexes (ULC) of heparin/PF4 and IgG bind to platelets via the platelet FcγIIa. The immune complexes are potent platelet activators leading to the release of platelet-derived procoagulant microparticles (Kelton and Warkentin, 2008).

PF4/heparin complexes form at stoichiometric molar ratios of 1:1 or 1:2 of PF4 and heparin. Excess heparin disrupts PF4/heparin complexes (Greinacher et al., 2008). In most clinical settings, UFH and LMWH are present in excess in the plasma. PF4 plasma concentrations also rise after intravenous heparin administration because it displaces PF4 from endothelial cells (Dawes et al., 1982). However, this increase is still below the stoichiometric concentration of PF4 that is optimal for complex formation. Optimal concentrations of PF4/heparin complex formation requires additional platelet activation for the plasma PF4 concentration to rise to the levels needed for the complex formation. These levels are reached in many patients undergoing major surgery (Visentin et al., 1996).

In general, there is a higher frequency of HIT with bovine heparin compared to porcine heparin (Schmitt and Adelman, 1993). The increased tendency for bovine heparin to induce HIT has been attributed to its higher degree of sulfation, which renders it better able to form an antigenic complex with PF4.

A high proportion (approximately 50%) of patients, especially those who have undergone cardiac surgery, will produce anti-PF4/heparin antibodies (Amiral et al., 1995,

Warkentin et al., 2000, Trossaert et al., 1998, Visentin et al., 1996). The resulting immune complexes cross-link FcγRIIa receptors on platelets and FcγRI on monocytes and cause activation. Cross-linking of the Fc receptors leads to platelet activation with the consequent release of platelet granular constituents (including PF4) and platelet microparticles, generation of thromboxane A_2, and ultimately platelet aggregation. This platelet activation releases PF4 from platelet α-granules allowing more PF4/heparin complexes to be formed and become bound to the platelet surface, creating more antigenic sites for the heparin-dependent anti-PF4/hepatin antibodies to bind. This creates a self-enhancing chain reaction that results in intense platelet activation and formation of platelet aggregates. The subsequent platelet activation increases thrombin production and creates a systemic hypercoagulable state that can culminate in venous and/or arterial thrombosis (Smythe et al., 2011).

1.4 Pathogenic and non-pathogenic anti-PF4/heparin antibodies in HIT patients

Although, autoantibodies against PF4/heparin complexes are formed in a high proportion of patients exposed to heparin, only a subset of these antibodies will activate platelets and cause HIT, referred to as pathogenic HIT antibodies (Trossaert et al., 1998, Nazi et al., 2015b). It has been shown that approximately 5 to 30% of patients who form HIT IgG antibodies will develop clinical HIT (Visentin et al., 1996). As a result, diagnosis of HIT is difficult due to the detection of clinically insignificant anti-PF4/heparin antibodies, also termed non-pathogenic antibodies.

Many investigations to understand the pathophysiology of HIT utilized KKO, a murine monoclonal antibody against human PF4/heparin complexes that causes thrombocytopenia and thrombosis in a transgenic mouse model (Arepally, 2000). KKO is used as a model pathogenic HIT antibody for antigenicity studies as it has been shown to compete with pathogenic HIT antibodies for binding to PF4 *in vitro* and form the ultra large immune complexes associated with HIT (Cuker et al., 2013). A previous mutagenesis study identified amino acids D7-Q9 as antigenic sites on PF4 for KKO (Cai et al., 2015). Another study found amino acids in the region between the 3rd and 4th cysteines (C36 and C52) to be important in KKO binding (Li et al., 2002). An X-ray crystallography study has shown KKO binds to specific amino acids on three monomers of the PF4 tetramer (Cai et al., 2015). Although studies of KKO are useful in providing models for the pathogenesis of HIT, patient sera need to be studied in order to fully define the complexity of the immune response in HIT.

Some studies have shown that the majority of HIT pathogenic antibodies share a similar binding site to PF4 as KKO; however, others indicate up to 10% of the HIT pathogenic antibodies are thought to have a different binding site (Cuker et al., 2013, Sachais et al., 2012, Kizlik-Masson et al., 2017). The polyspecific immune response in HIT patients further complicates studies (Nazi et al., 2015b, Ziporen et al., 1998, Suh et al., 1998, Li et al., 2002) and it has been observed that there is considerable overlap between true-positive HIT (EIA-positive/SRA-positive; pathogenic) and false-positive (EIA-positive/SRA-negative; non-pathogenic) patient samples. Our lab has previously

observed that 36% of EIA-positive/SRA-negative (false-positive) HIT samples contain subthreshold levels of platelet-activating antibodies using the PF4-SRA, a modified SRA involving the addition of exogenous PF4 (Nazi et al., 2015b).

1.5. The immune response in HIT

The immune response of HIT has several atypical features. In patients who receive heparin for the first time (as early as 4 days) there is a rapid formation of anti-PF4/heparin IgG antibodies. The IgG antibodies are the predominant class of HIT antibodies formed, without a preceding IgM (Greinacher et al., 2009, Warkentin et al., 2009). Up to 50% of patients after cardiopulmonary bypass surgery and 20-30% of orthopedic surgery patients (many of whom have not been previously exposed to heparin) develop anti-PF4/heparin IgG antibodies in the second week after heparin exposure (Greinacher et al., 2005, Selleng et al., 2010b, Warkentin et al., 2000). This is not characteristic of a primary immune response, where there is usually a predominant formation of IgM antibodies followed by a later and weaker response of IgG.

The immune response in HIT has further interesting features consistent with an evolutionary ancient type of immune response. There is no clear restriction to IgG, but IgM and IgA antibodies are generated at the same time (Greinacher et al., 2009, Warkentin and Kelton, 2001). However, the antibody titers rapidly decrease within several weeks. There is also a lack of memory B cells for anti-PF4/heparin IgG in patients who developed anti-PF4/heparin antibodies after cardiac surgery (Selleng et al., 2010a).

Nguyen et al. (2017) have now also found increasing evidence that in a subset of patients the anti-PF4/heparin antibodies are autoantibody-like, as they recognize PF4 bound to platelets even in the absence of pharmacologic heparin.

1.6 Clinical features of HIT

In HIT, the platelet count typically begins falling 5-10 after the immunizing exposure to heparin. Once the platelet fall begins, it usually declines by at least 50% over the next few days. In 25-30% of cases though, the onset of thrombocytopenia occurs quickly, within 24 hours of heparin administration (or an increase in dose) and is its own subclass of HIT – rapid-onset HIT (Warkentin, 2003). These patients have been studied and had often been exposed to heparin within the past 100 days. Because they already have anti-PF4/heparin antibodies circulating, thrombocytopenia promptly appears. The ensuing thrombocytopenia moderately severe but platelet counts rarely falls below 10 x 10^9/L (Warkentin, 2003).

Despite the presence of thrombocytopenia, thrombotic complications are common in patients with HIT, occurring in up to 70% of patients (Girolami et al., 2003). The thrombotic complications can be venous, arterial, and/or microcirculatory. Lower limb deep vein thrombosis (DVT) is the most common HIT-associated thrombotic complication. Bilateral DVT and pulmonary embolus are also relatively common (Warkentin, 2015). The venous thrombosis in HIT can be extensive and result in limb gangrene or *phlegmasia cerulea dolens*, a condition extremely uncommon in patients

without HIT. Arterial thrombosis in HIT usually involves the distal aorta and lower limb arteries causing limb ischemia, which may lead to limb gangrene and leg amputation (Weismann and Tobin, 1958, Rhodes et al., 1973). Less commonly, arterial thrombosis in HIT results in thrombotic stroke, acute myocardial infarction, or occasionally to occlusions of the brachial, mesenteric, and renal arteries.

1.7 Current practices in HIT diagnosis

Currently, the diagnosis of HIT requires analyzing both clinical and laboratory data to reach an accurate conclusion (Otis and Zehnder, 2010). The immune response in HIT requires several days until antibodies are produced in sufficient quantities to cause platelet activation. HIT should be considered when there is otherwise unexplained thrombocytopenia and/or thrombosis in patients receiving heparin. HIT can also be considered in patients who have thrombocytopenia or new thrombosis and have received heparin in the last 2 weeks.

One of the first steps used is the clinical 4Ts test probability score (Warkentin et al., 2015). This test determines the probability of HIT according to the degree and timing of thrombocytopenia, the presence of thrombosis, and the likelihood of other causes of thrombocytopenia. The scoring systems are especially useful at ruling out a diagnosis of HIT because a low score indicates a low (<2%) probability of having a positive platelet activation assay. In contrast, even when the scoring system yields a high score, the probability of HIT being confirmed by a positive platelet activation test is only

approximately 50%. The positive predicted value is low for the 4Ts test and is subject to variation based on the observer (Warkentin et al., 2015).

HIT is a clinico-pathologic disorder where a clinical diagnosis should be confirmed with the laboratory platelet activation test (Figure 1.2). Two types of serological laboratory tests can be ordered and used to confirm a HIT diagnosis. Functional assays, such as the ^{14}C-serotonin release assay (SRA) or the heparin-induced platelet activation assay (HIPA) are accepted as the gold standard in diagnosing HIT and have a high specificity and sensitivity (Gupta et al., 2015, Greinacher et al., 1991, Sheridan et al., 1986). However, these tests are technically demanding and are available only in a small number of reference laboratories.
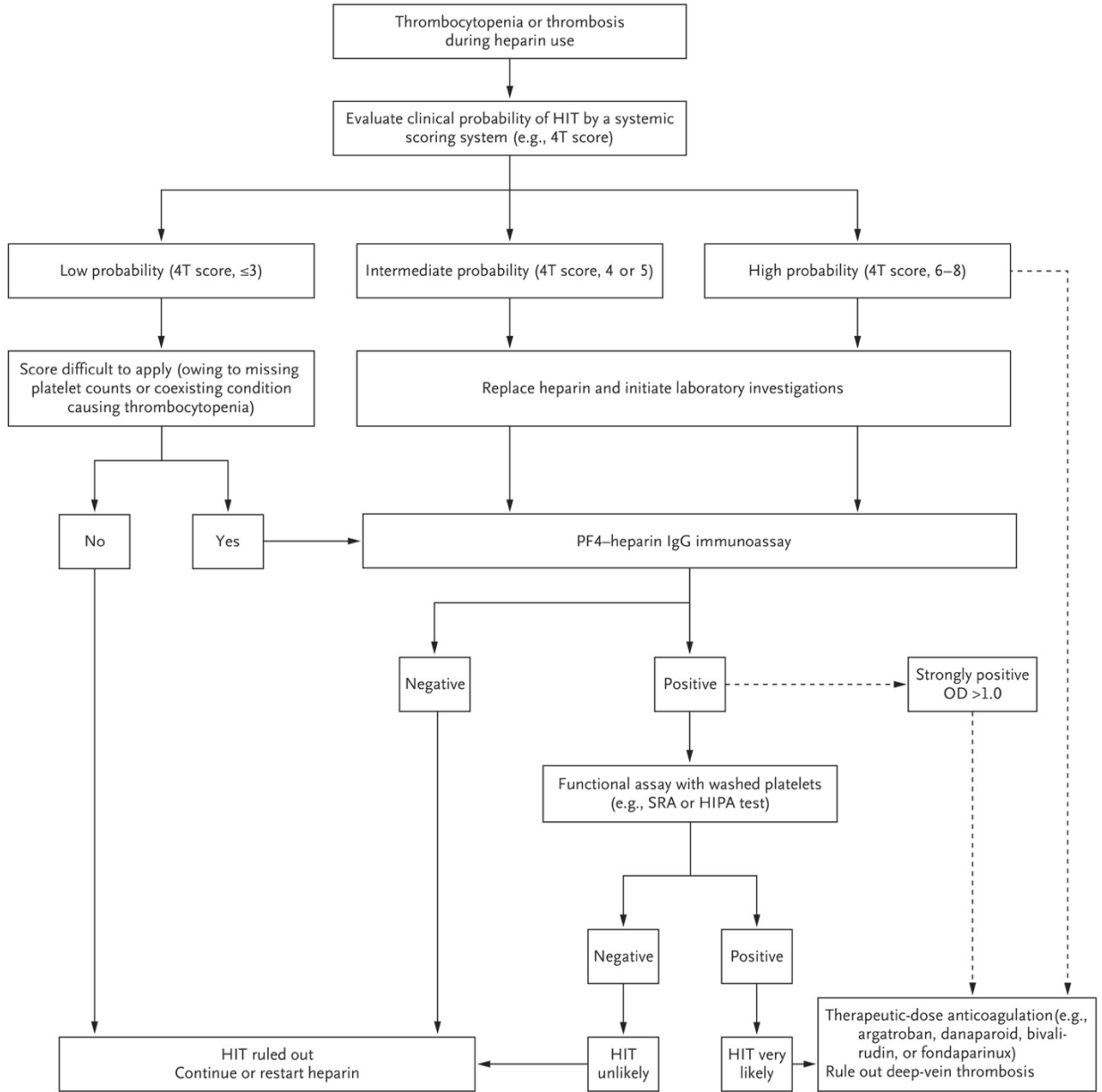
Figure 1.2: Diagnosis of HIT. The flowchart provides a guide to decision making regarding a patient who is suspected to have HIT. Enzyme immunoassays (EIAs) for PF4/heparin antibodies are widely available, whereas functional assays with the use of washed platelets, such as the serotonin-release assay (SRA) or the heparin-induced platelet-activation (HIPA) test, are restricted to specialized laboratories (Greinacher, 2015).

The more commonly used PF4/heparin enzyme immunoassays (EIAs) are more available and simpler to perform but are not as effective as the functional assays due to low sensitivity (Nagler et al., 2016). This high rate of false positives is a result of the EIA being unable to distinguish between pathogenic and non-pathogenic antibodies, leading to a frequent overdiagnosis of HIT unless it is confirmed by the SRA or HIPA functional tests (Warkentin, 2011a, Kelton, 2002, Cuker, 2011). All anti-PF4/heparin antibodies that are screened will be positive in the EIA (EIA+), however, only anti-PF4/heparin antibodies that activate platelets will be positive in the SRA and these have a high correlation with HIT (Figure 1.3).
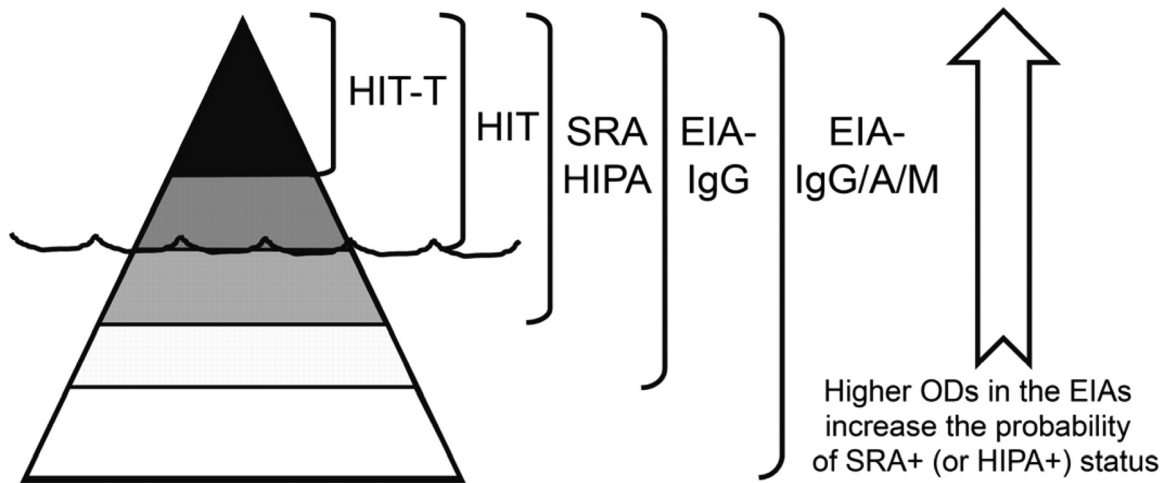
Figure 1.3: Iceberg model of HIT. Clinical HIT, comprising HIT with or without thrombosis, is represented by the portion of the iceberg above the waterline. Three types of assays are highly sensitive for the diagnosis of HIT: the washed platelet activation assays (SRA and HIPA), the IgG-specific PF4-dependent EIAs (EIA-IgG), and the polyspecific EIAs that detects anti-PF4/heparin antibodies of the 3 major immunoglobulin classes (EIA-IgG/A/M). In contrast, diagnostic specificity varies greatly among these assays where the platelet activation assays (SRA and HIPA) have the highest specificity and specificity is lower for the EIA-IgG/A/M. This is because the EIA-IgG/A/M is most

likely to detect clinically irrelevant, non-platelet-activating anti-PF4/heparin antibodies (Warkentin, 2011b).

The large discrepancy between the two diagnostic methods of HIT, the functional assays and the EIA, indicates that there are differences between anti-PF4/heparin antibodies that activate platelets (pathogenic antibodies, EIA+/SRA+) and those that do not (non-pathogenic antibodies, EIA+/SRA-)(Nazi et al., 2015b). There is currently no rapid diagnostic test for HIT with high specificity, making the development of such an assay of crucial importance. In order to differentiate between pathogenic and non-pathogenic antibodies, a better understanding of the epitope(s) on PF4 is needed.

1.8 Platelet factor 4 (PF4)

Understanding the structure of PF4 is important in order to understand its crucial role in the pathophysiology of HIT. PF4 is a member of CXC chemokines group, where two cysteines are separated by a single amino acid (Deuel, 1977). Most of the CXC chemokines have an ELR motif at the N-terminus, which plays a major role in inflammation. The full gene sequence of PF4 is made up of 100 amino acids where the initial 30 amino acids encodes for a hydrophobic signal portion (Deuel, 1977). When processed, the mature monomeric human PF4 chemokine contains 70 amino acids and is approximately 7.8 kDa that lacks the ELR motif (Deuel, 1977).

PF4 has no tryptophan or methionine residues. PF4 contains three aromatic amino acids: His-23, His-35, and Tyr-60. Found within PF4 are four cysteine residues, which

form two disulfide bonds (Zhang et al., 1994). The amino-terminal contains a glutamate rich sequence and the middle of the protein sequence contains a relatively hydrophobic region containing three anti-parallel ß-strands (Figure 1.4A). Found at the carboxy-terminus is an α-helical amphiphatic structure containing two pairs of adjacent lysines separated by two amino acids (Figure 1.4A)(Zhang et al., 1994). The amino-terminus is an unstructured loop covalently constrained to the ß-sheet domain by the two disulfide bridges. The monomeric structure of PF4 greatly resembles other chemokines within the CXC family but the PF4 quaternary structure is more divergent (Park et al., 1990). PF4 exists as an equilibrium between monomer, dimer, and the more predominant tetramer (Mayo and Chen, 1989). The tetramer comprises of two asymmetric dimers, forming a cylindrical structure composed of an equatorial ring of positively charged amino acids of the carboxy-terminal α-helices and internal cationic amino acids (Warkentin and Sheppard, 2006).
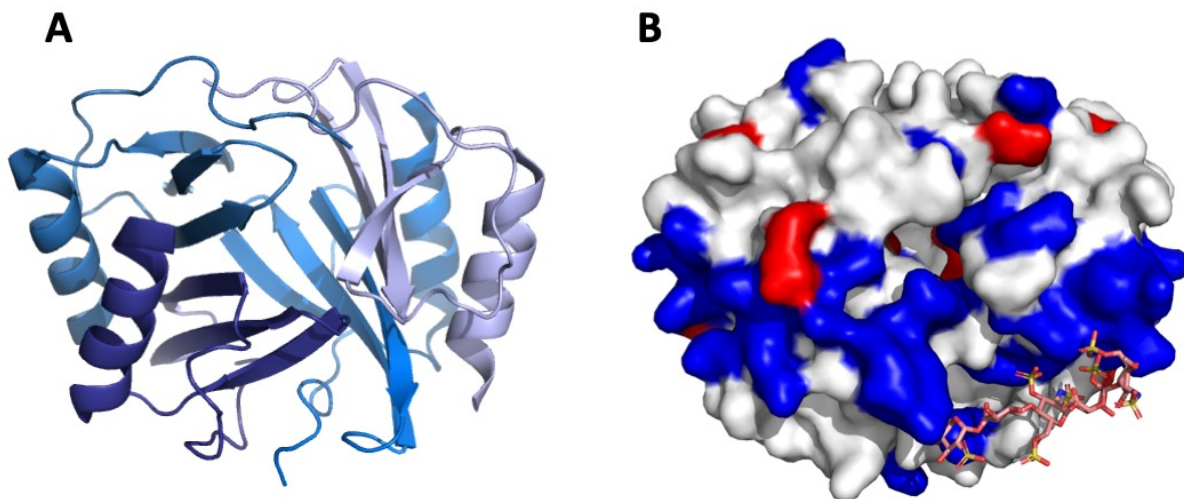
Figure 1.4: Structure of PF4. A) Cartoon representation of overall structure of PF4. Each monomer is coloured a different shade of blue. B) Surface representation of PF4 and fondaparinux where positively charged amino acids (histidine, arginine, and lysine) are coloured blue and negatively charged amino acids (aspartic acid and glutamic acid) are coloured red. Images generated in PyMOL using PDB 4R9W (Cai et al., 2015, Zhang et al., 1994).

When platelets are activated, PF4 is released from platelet α-granules with platelet basic proteins, tissue activating peptides, fibrinogen, and growth factors. Human plasma concentrations of PF4 are usually low, containing approximately 2-10 ng/mL (Files et al., 1981). Upon platelet activation, the PF4 concentrations in the blood increase to 5-10 μg/mL. This increase in concentration greatly promotes activation of additional platelets and monocytes.

1.9 PF4 binding to heparin

PF4 binds to heparin with the following amino acids: R20, R22, H23, T25, K46, R49, K61, K62, K65, K66 (Figure 1.4B)(Mayo et al., 1995). When PF4 binds heparin, it undergoes a conformational change. In this interaction, linear, ridge-like protein

complexes are formed, which decreases the distance between each PF4 tetramer (Greinacher et al., 2006). Nuclear magnetic resonance (NMR) studies have shown that when PF4 complexes with heparin, PF4 becomes partially unfolded (Mikhailov et al., 1999). Binding of anti-PF4/heparin antibodies to PF4/heparin complexes require certain characteristics to be met. To be antigenic, when PF4 binds to heparin, it must undergo a >30% increase in antiparallel β-sheet content and a negative change in enthalpy larger than -4000 cal/mol$_{PF4}$ (Kreimann et al., 2014, Brandt et al., 2014). These conditions are only met when the heparin chain length is at least 12 saccharides in length. In addition, separate mutagenesis studies have shown that at least two neoepitopes are formed upon binding of PF4 with heparin (Li et al., 2002). As a result, further analysis and study is required to verify some of the predictions and learn more of the conformational change heparin induces upon PF4.

1.10 Platelet surfaces and its role in HIT

The epitope of anti-PF4/heparin antibodies is conformation sensitive, and additional polyanions on the platelet surface might influence the conformation of PF4 and PF4/heparin complexes or their three-dimensional presentation (Padmanabhan et al., 2015a). Chondroitin sulfate has been identified as the most abundant GAG found on platelet surfaces, followed by heparan sulfate (Nader, 1991). Surface-bound PF4 is antigenic for HIT antibodies and KKO over a narrow range of PF4 concentrations, leading to platelet activation through FcγRIIA (Rauova et al., 2006). It has been suggested that PF4 forms antigenic complexes with endogenous GAGs on the surface of

platelets similar to ultra large complexes that form between UFH and PF4 in solution (Rauova et al., 2005). However, it has also been shown that complexes of PF4 with the GAGs on the platelet surface either exposes a different epitope or allows for better access of pathogenic HIT antibodies to its epitope (Nguyen and Greinacher, 2017). Currently, it is unresolved which additional binding partners on platelet surfaces interfere with the conformational change or cause different presentations of PF4/polyanion complexes.

1.11 Thesis Objectives

That last two decades have identified key components in the binding of PF4 to pathogenic HIT antibodies. Recent structural and biochemical characterization has revealed some amino acids that could make up the epitope of pathogenic HIT antibodies and the different classes of anti-PF4/heparin antibodies that exist. However, an exact definition of the epitopes on PF4 for pathogenic and non-pathogenic anti-PF4/heparin antibodies remains an active area of research.

We propose that specific amino acids of PF4 make up the pathogenic HIT antibody epitope. Discovering the amino acids important for this interaction can differentiate pathogenic from non-pathogenic HIT antibodies and characterize why some antibodies are platelet-activating whereas others are not. With this information, we would like to develop a new diagnostic tool that exploits mutations of PF4 epitopes to definitively diagnose patients with HIT. In order to determine which amino acids make up the interaction between PF4 and the HIT antibodies, alanine scanning mutagenesis was

performed on both EIA-positive/SRA-positive and EIA-positive/SRA-negative patient samples. These results have helped identify some of the amino acids in PF4 that likely play a role in the antibody interaction. Since the start of my PhD, we have started to use this information to not only develop a new diagnostic tool but also learn more of the assembly of the HIT antigenic complex. In particular, the work included in this thesis will address the three following objectives:

    (i)   Develop a purification method for recombinant PF4 that can be applied to future PF4 mutants to study the epitopes of pathogenic HIT antibodies (chapter 2).

    (ii)   Determine amino acids of PF4 that are important in the epitope of pathogenic HIT antibodies using alanine scanning mutagenesis of PF4 (chapter 3).

    (iii)   Describe the necessary features in order for an anti-PF4/heparin antibody to cause platelet activation and lead to HIT (chapter 4).

    (iv)   Elucidate the role fluid-phase HIT immune complexes play in activating platelets (chapter 5).

There will be overlap between chapters as similar introductions and methods are used in different studies and some chapters are reprints of manuscripts.

Chapter 2

Development of a high yield expression and purification system for platelet factor 4

2.1 AUTHOR'S PREFACE

This chapter is focused on developing a purification protocol for recombinant PF4. Downstream biochemical analysis of the pathogenic HIT antibodies against PF4 will require large amounts of PF4 to perform the future planned assays. Recombinant PF4 expressed by bacteria produces increased soluble yields with longer induction times at lower temperatures. However, additional detergents are required to solubilize PF4 during the lysis of bacteria for affinity purification. By testing different growth and lysis conditions, I maximized the amount of PF4 that could be purified using a bacterial expression system (Figure 2.1 and 2.2). I checked oligomerization of the recombinant PF4 using cross-linking experiments and size exclusion chromatography (Figure 2.3 and 2.4). J.C. Moore and J.W. Smith performed enzyme immunoassays and PF4-SRAs respectively to compare recombinant PF4 to platelet-derived PF4 (Figure 2.5 and 2.6). J.C. Moore, J.W. Smith and I analyzed the data and prepared the figures. Dr. I. Nazy, Dr. D.M. Arnold, Dr. J.G. Kelton and I wrote the manuscript.

2.2 ABSTRACT

Heparin-induced thrombocytopenia (HIT) is an adverse drug reaction characterized by IgG antibodies bound to complexes of platelet factor 4 (PF4) and heparin. The majority of diagnostic tests for HIT rely on an exogenous source of PF4 to identify anti-PF4/heparin antibodies. These include the PF4-dependent enhanced serotonin release assay (PF4-SRA) among others. Using a bacterial expression system, we developed a novel and efficient method of producing recombinant human PF4 (rhPF4) that is biochemically and antigenically similar to platelet-derived human PF4. rhPF4 was produced using the pET expression system in the BL21(DE3) strain of *Escherichia coli*. The system was optimized for protein expression using isopropyl β-D-1-thiogalactopyranoside at different induction temperatures and incubation times. rhPF4 solubility was improved by using different detergents during cell lysis and by purifying with heparin affinity and ion exchange chromatography. Biochemical characteristics of rhPF4 were investigated using mass spectrometry, SDS-PAGE analysis and size exclusion chromatography and compared to platelet-derived PF4. Antigenic and functional characteristics of rhPF4 were studied using the anti-PF4/heparin EIA and the PF4-SRA. Using this method, we could produce 11.4 ± 0.6 mg of pure rhPF4 per liter of bacterial culture. Absorbance readings from the anti-PF4/heparin EIA using platelet-derived and rhPF4 were highly correlated (n=194; r = 0.9545, p < 0.0001); and functional release of serotonin in the PF4-SRA induced by anti-PF4/heparin antibodies was similar with either platelet-derived or rhPF4 and heparin (r = 0.9597, p < 0.0001). Our method of rhPF4 production is efficient and does not rely on a source of platelets. The rhPF4

purification method described produces greater yields at a lower cost than other current methods. The application of this method can improve the efficiency of biochemical investigations and HIT diagnostic testing by supplying sufficient amounts of PF4.

2.3 INTRODUCTION

Platelet factor 4 (PF4, CXCL4) is a member of a large group of CXC chemokines, in which two cysteines are separated by a single amino acid (Deuel, 1977). This family of chemokines also includes interleukin-8 and CXCL (Warkentin, 2011a). PF4 is involved in numerous biological processes by acting on a broad spectrum of different cell types. These processes include regulation of angiogenesis, megakaryopoiesis, and activation or proliferation of leukocytes (Lambert et al., 2007, Koenen et al., 2009, Engstad et al., 1995). PF4 is one of the most abundant proteins released during platelet activation (Files et al., 1981).

The fully processed protein sequence of mature monomeric human PF4 is comprised of 70 amino acids and is approximately 7.8 kDa (Deuel, 1977). The amino-terminal contains a glutamate rich sequence and the middle of the protein sequence contains a relatively hydrophobic region with three anti-parallel ß-strands (Zhang et al., 1994). Found at the carboxy-terminal is an α-helical amphiphatic structure containing two pairs of adjacent lysines separated by two amino acids. The primary structure of PF4 greatly resembles other chemokines within the CXC family, but the quaternary structures are more divergent. PF4 exists as an equilibrium between monomer, dimer, and tetramers,

with the predominant species being tetramer (Mayo and Chen, 1989). The tetramer is comprised of two asymmetric dimers, and forms a cylindrical structure composed of an equatorial ring of positively charged amino acids (Warkentin, 2011a). This ring is the binding site for heparin and other glycosaminoglycans.

PF4 plays a critical role in the pathogenesis of heparin-induced thrombocytopenia (HIT), an adverse drug reaction (Warkentin and Sheppard, 2006). HIT is a prothrombotic disorder that can lead to amputation, stroke, and death in as many as 20-30% of patients (Ahmed et al., 2007, Greinacher, 1995). When heparin is administered, it binds to PF4 released from activated platelets and forms immunogenic multimolecular PF4/heparin complexes. In patients who develop HIT, the complexes bind antibodies against PF4/heparin, which cross-link FcγIIa receptors on platelets and FcγRI on monocytes, releasing procoagulant materials, which cause the syndrome (Smythe et al., 2011).

The accurate and rapid diagnosis of HIT is essential (Cuker, 2011). Several types of assays are used in laboratory testing for HIT, such as the enzyme-immunoassays (EIAs) and functional assays, such as the serotonin release assay (SRA) and the heparin-induced platelet aggregation (HIPA)(Sheridan et al., 1986, Greinacher et al., 1991). The PF4-SRA (Nazi et al., 2015b) and the P-selectin Expression Assay (PEA)(Padmanabhan et al., 2016) are relatively new tests that have been developed to measure platelet activation. These assays require large amounts of PF4, which is often the limiting step in

upscaling. New efficient methods of producing larger amounts of pure and active PF4 are needed.

In this report, we describe a novel method of producing recombinant bacteria-derived human PF4 (rhPF4) that exhibits identical biochemical characteristics and antibody-binding properties as platelet-derived PF4. This purification protocol takes advantage of an established *E. coli* expression system to maximize yield.

2.4 MATERIALS AND METHODS

Construct design and optimization of recombinant PF4 expression

The full length coding sequence of human PF4 (Deuel, 1977) was cloned into the pET22b expression vector using restriction sites NdeI and HindIII (GenScript, Piscataway, NJ, USA). The pET22b-rhPF4 expression construct was then introduced into *E. coli* BL21(DE3) cells through heat-shock transformation (Bergmans et al., 1981). Expression and solubility of rhPF4 was assessed using small-scale expression cultures. An overnight culture of the pET22b-rhPF4 expression construct in Luria-Bertani (LB) media supplemented with 100 µg mL^{-1} of ampicillin was diluted 1:50 in LB media with 100 µg mL^{-1} of ampicillin and grown with shaking at 225 rpm and 37°C to mid-exponential phase (OD$_{600nm}$ = 0.6). Expression of rhPF4 was induced with 0.5 mM isopropyl-D-1-thiogalactopyranoside (IPTG) and cultures were then grown with shaking at 225 rpm and 37°C for 3 hours, 25°C for 5 hours, or 16°C overnight. Following centrifugation (3,500x*g*) and removal of excess media, cell pellets were resuspended in 20

mM sodium phosphate, pH 7.2, 200 mM NaCl, 1.4 mM β-mercaptoethanol and lysed

using combinations of different detergents including: 1% lauryldimethylamine-oxide

(LDAO), 1% Triton X-100, 0.5% deoxycholic acid (DOC), 0.5% 3-((3-cholamidopropyl)

dimethylammonio)-1-propanesulfonate (CHAPS), and/or 0.5% sodium dodecyl sulfate

(SDS). Protein expression and solubility were assessed for each of the growth conditions

using 18% SDS-PAGE.

Large-scale expression and purification of recombinant PF4 in E. coli

Large scale expression of rhPF4 was achieved by growing the pET22b-rhPF4

expression construct in LB media with 100 µg mL^{-1} of ampicillin with shaking at 225 rpm

and 37°C to mid-exponential phase (OD$_{600nm}$ ≈ 0.6), before inducing protein production

with 0.5 mM IPTG. Once induced, cultures were then grown with shaking at 225 rpm and

25°C for 5 hours and then harvested by centrifugation (3,300xg). Three grams of cell

pellet wet weight were resuspended in 40 mL lysis buffer (20 mM sodium phosphate, pH

7.2, 400 mM NaCl, 1.4 mM β-mercaptoethanol, 5% (v/v) glycerol, 1% (v/v) Triton X-

100 (Thermo Scientific Inc., Burlington, ON, CAN), and 0.5% (v/v) DOC (Sigma-

Aldrich, Oakville, ON, CAN) with protease inhibitors (Roche cOmplete EDTA free

protease inhibitor tablets, Roche, Laval, QC, CAN), lysed by sonication consisting of

three 30 second pulses with a 30 second cooling interval between pulses, and incubated

with an additional 2 mM MgCl$_2$ and 10 µg mL^{-1} DNaseI for 30 min on ice. The

supernatant was then cleared by centrifugation at 40,000xg for 40 min and applied onto a

HiTrap Q HP column (GE Healthcare, Mississauga, ON, CAN) equilibrated with 20 mM

sodium phosphate, pH 7.2, 400 mM NaCl, 1.4 mM β-mercaptoethanol, and 5% (v/v) glycerol. The unbound fraction from the Q HP column, containing the rhPF4, was then incubated at 4°C, overnight. The sample was then diluted by 2-fold with 20 mM sodium phosphate, pH 7.2, 1.4 mM ß-mercaptoethanol, and 5% (v/v) glycerol to yield a NaCl concentration of 200 mM. The final solution was filtered with a 0.2 µm filter (Pall Acrodisc, St. Laurent, QC, CAN) and loaded onto a HiTrap Heparin HP column (GE Healthcare, Mississauga, ON, CAN) equilibrated with 20 mM sodium phosphate, pH 7.2, 1.4 mM ß-mercaptoethanol, 5% (v/v) glycerol, and 200 mM NaCl. Contaminants were eliminated with a 10 column volume 0.5 M NaCl wash step and rhPF4 was eluted with a linear gradient from 0.5 M to 2 M NaCl over 70 minutes. Fractions were collected and analyzed by 18% SDS-PAGE and the ones containing pure rhPF4 were pooled, concentrated, and the buffer was exchanged to 1.5 M NaCl/PBS using an Amicon Ultra-15 3kDa MWCO centrifugal filter (EMD Millipore, Etobicoke, ON, CAN). The concentration of rhPF4 was determined using the bicinchoninic acid assay (BCA, ThermoScientific, Burlington, ON, CAN).

Biochemical Properties of Recombinant PF4

To assess the oligomerization state and purity, the purified rhPF4 was analyzed on a Superdex 75 10/300 GL (GE Healthcare, Mississauga, ON, CAN) equilibrated with 1.5M NaCl/PBS buffer. Platelet-derived PF4 or rhPF4 (0.25 mg) was loaded onto the Superdex 75 and both elution profiles were measured at an absorbance of 230 nm. In addition, 50 µg of rhPF4 and platelet-derived PF4 were sent to the Mass Spectrometry

Facility at the SPARC Biocentre at the Hospital for Sick Children in Toronto for liquid chromatography-mass spectrometry (LC-MS) to determine and compare the primary amino acid sequences of both proteins. 80 µg of rhPF4 and platelet-derived PF4 were also sent to the McMaster University Biointerfaces Institute for matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) to determine the molecular mass of each PF4. To assess the ability of the rhPF4 to form tetramers, platelet-derived PF4 and rhPF4 (50 µM) were incubated with increasing concentrations of Bis(sulfosuccinimidyl)suberate (BS^3, Sigma Aldrich, Oakville, ON, CAN) from 0-400 µM in 20 mM HEPES, pH 7.2 and 300 mM NaCl for 30 minutes at ambient temperature. Crosslinking reactions were quenched with 100 mM Tris, pH 7.0 for 15 minutes at ambient temperature and resolved on 4-15% SDS polyacrylamide gels (Bio-Rad, Mississauga, ON, CAN) stained with Coomassie Brilliant Blue.

Anti-PF4/Heparin Enzyme-immunoassay

The antigenic and binding characteristics of rhPF4 was investigated using an in-house anti-PF4/heparin IgG-specific EIA (Horsewood et al., 1996, Warkentin and Sheppard, 2006). 96-well Maxisorp plates (Thermo Fisher Scientific, Waltham, MA, USA) were precoated with PF4 (rhPF4 or platelet-derived PF4) and heparin (60 µg mL^{-1} PF4 and 1 IU mL^{-1} UFH diluted in 50 mM bicarbonate-carbonate buffer, pH 9.6) and incubated overnight at 4°C. After washing with PBS+0.05% Tween 20 and PBS, the plates were blocked with 200 µL/well 3% bovine serum albumin at ambient temperature for 2 hours. Following a wash step, 100 µL/well of 1/50 dilutions of patient sera from

SRA-positive (EIA-positive and SRA-positive, n=27), SRA-negative (EIA-positive and SRA-negative, n=54), or sera from healthy donors or patients that tested EIA-negative (n=113), were added to duplicate wells and incubated for 1 hour at ambient temperature. Samples from patients suspected of having HIT were initially tested with an in-house anti-PF4/heparin IgG EIA and the commercial PF4 enhanced assay (Immucor, Inc., Norcross, GA, USA). Sera that tested >0.45 in the IgG EIA were considered as EIA-positive and were also tested in the SRA. EIA-positive samples that had a >20% serotonin release in the SRA as described by Sheridan et al., 1986(Sheridan et al., 1986) were considered positive and denoted as SRA-positive. The EIA-positive samples that did not test positive in the SRA were denoted as SRA-negative. After washing, 100 µL of alkaline phosphatase-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) was added to wells at a 1/4000 dilution, followed by the addition of the substrate, p-nitrophenylphosphate in 10 mM diethanolamine, 0.5 mM $MgCl_2$ (pH 9.6), to detect bound anti-human IgG antibodies. The OD_{405} was used to assess the presence ($OD_{405} \geq 0.45$; EIA-positive) or absence ($OD_{405} <$ 0.45; EIA-negative) of anti-PF4/heparin IgG antibodies in sera. Positive and negative control samples were included on each test plate. The absorbance at 405 nm with reference wavelength of 490 nm was measured using a TECAN Sunrise plate reader (TECAN Group Ltd., Zurich, Switzerland).

The PF4-Serotonin release assay

To ensure the preservation of functional platelet activation upon HIT antibody

binding, we compared results of a functional test, the PF4-SRA using either platelet-derived PF4 or rhPF4. The PF4-SRA is similar to the standard SRA, but with the addition of exogenous PF4 instead of heparin (Nazi et al., 2015b). Platelets were labelled with ^{14}C-serotonin and prepared as described for the standard SRA and incubated with sera from SRA-positive (EIA-positive and SRA-positive, n=29) or SRA-negative (EIA-positive and SRA-negative, n=10) patients in the presence of exogenous PF4 (0–100 μg mL^{-1}) in microtiter plates (Nunc Maxisorp, Thermo Fisher Scientific Inc., Burlington, ON, CAN). Release data shown only for 50 μg mL^{-1} PF4. After a 1 hour incubation with shaking, PBS-EDTA (100 μL) was added and the plates were centrifuged. An aliquot of supernatant (50 μL) was removed from each well and added to scintilant in microtiter plates (Immulon, Thermo Scientific Inc., Burlington, ON, CAN), and the release of ^{14}C-serotonin was measured in a scintillation counter (Packard, Topcount, Meriden, CT, USA). The quantity of ^{14}C-serotonin release in each well was determined as described for the standard SRA (Sheridan et al., 1986).

Statistical Analysis

Anti-PF4/heparin EIA results are reported as a mean of duplicate trials. A Pearson correlation test was applied to the data from the anti-PF4/heparin EIA and the PF4-SRA comparisons to compare the results between platelet-derived PF4 and rhPF4. All data analysis was performed using GraphPad 6 (GraphPad Prism, San Diego, CA, USA). A *p* < 0.05 was considered statistically significant.

2.5 RESULTS

Optimization of recombinant PF4 expression

To optimize the expression of rhPF4 using the BL21(DE3) *E. coli* expression system, small-scale expression tests were performed at different temperatures and induction methods. Induction at mid-exponential cell growth with 0.5 mM IPTG for 5 hours at 25°C produced the largest amount of soluble PF4, based on visual assessment (Figure 2.1). These conditions were used for the large-scale expression of rhPF4 in 1 L of bacterial culture. Different detergent combinations were also tested for optimal lysis and yield of rhPF4 and the combination of 1% Triton X-100 and 0.5% DOC was selected. The yield of rhPF4 after performing the optimized purification from 1 L of *E. coli* was approximately 11.4 ± 0.6 mg (n = 4 separate purifications) and migrated as a single band with a relative mobility of approximately 8kDa when assessed by Coomassie Brilliant Blue stained SDS-PAGE (Figure 2.2).
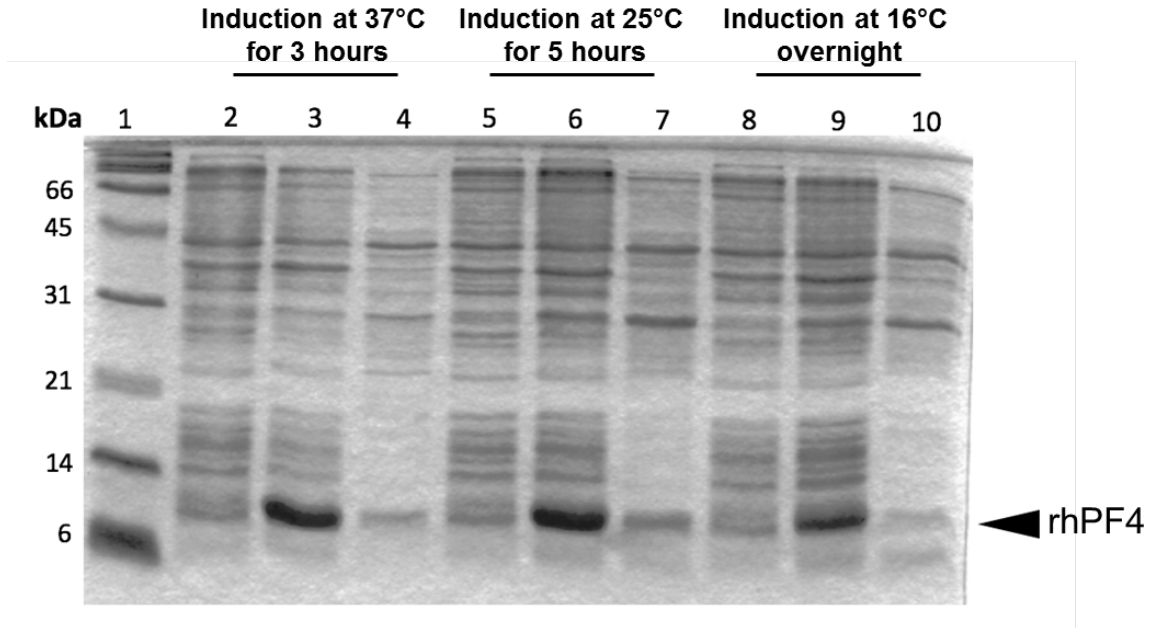
Figure 2.1: **Testing Expression and Solubility of PF4-BL21 at Different Induction Temperatures and Durations**. Expression and solubility of PF4 in BL21 (DE3) with a small-scale lysis. Lane 1 represents protein ladder standards. Lanes 2-4 represent samples induced at 37°C for 3 hours. Lanes 5-7 show the results for samples induced at 25°C for 5 hours. Lanes 8-10 represent samples induced at 16°C and grown overnight. Lanes 2, 5 and 8 denote the time point in the culture at mid-exponential phase before the addition of 0.5 mM IPTG. Lanes 3, 6 and 9 denote the time point of the culture growth after the addition of 0.5 mM IPTG at the end of their respective induction temperatures. Lanes 4, 7 and 10 denote the proteins found in the supernatant after small-scale lysis, indicating soluble proteins. The condition that best optimized solubility and expression of rhPF4 from BL21 (DE3) is by inducing the culture for 5 hours at 25˚C, based on visual assessment.

The yield and purity of the rhPF4 using this method was compared to yields of human PF4 purified from outdated platelets, that we performed in parallel to the newly developed system (Levine and Wohl, 1976). The yield of rhPF4 from 1 L of bacterial culture using the novel method was similar to PF4 produced from 50 units of outdated human platelets (approximately 10 mg)(Levine and Wohl, 1976)(Table 2.1). This method

of rhPF4 purification produces yields for many further biochemical studies including X-

ray crystallography which demands large amounts of protein.
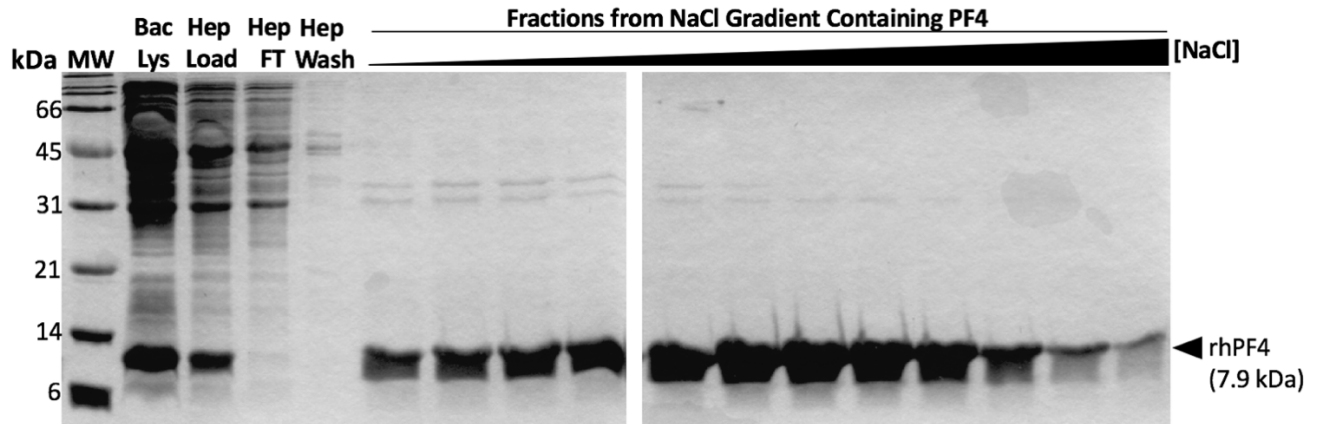
Figure 2.2: Purification of rhPF4 from E. coli using Affinity Chromatography.
Figure shows the corresponding SDS-PAGE for the heparin column purification of
recombinant PF4. MW represents protein ladder standards. Bac Lys lane shows the
bacterial lysate after lysis with sonication and before anion exchange chromatography
with the Q Sepharose column. Hep Load is the sample after the Q Sepharose column and
what is loaded onto the Heparin affinity chromatography column. Hep FT is the sample
of proteins that do not bind to the Heparin Sepharose column. Hep Wash represents the
proteins removed from the column at 0.5 M NaCl. The remaining fractions are from the
NaCl gradient at 1.6-1.8 M NaCl showing the fractions that contain rhPF4. The
arrowhead indicates protein band that corresponds to rhPF4 (approx. 7.9 kDa). All protein
samples were run on a denaturing 18% SDS-polyacrylamide gel.

**Table 2.1: Comparison of eukaryotic and *E. coli* systems for recombinant PF4
expression and purification.**

Construct	Expression	Purpose	Purification	Purity and/or Yield	Reference
pIN-III-ompA-2 (+7aa N-term and +4aa C-term, N→D47)	*E. coli*	Immunoregulatory studies	Heparin-agarose affinity chromatography followed by reverse-phase HPLC	90% purity	Barone et al., 1988(Barone et al., 1988)
pT7-7 (+1M)	*E. coli* K38	Biologic function confirmation	Heparin agarose affinity chromatography	>95% purity and approx. 450 µg L^{-1}	Park et al., 1990(Park et al., 1990)

			followed by reverse-phase C18 HPLC		
pREV2 (+42aa N-term fusion moiety)	*E. coli* SG20251	Biological activities	Guanidine extraction, CM-sepharose, CNBr cleavage, S-sepharose, finished with reverse-phase HPLC	118000 µg L^{-1}	Myers et al., 1991(Myers et al., 1991)
pT7-7 (+1M)	*E. coli* BL21 pLysS	Determining antigenic epitopes on PF4	Heparin agarose affinity chromatography followed by reverse-phase C18 HPLC	N/A	Ziporen et al., 1998(Ziporen et al., 1998)
pMT/BiP/V5-His	Drosophila expression system	Crystallography	Filtered and then used heparin affinity chromatography	N/A	Sachais et al., 2012(Sachais et al., 2012)
pET22b, no 6xHis-tag	*E. coli* BL21	Biochemical testing	Heparin-agarose affinity chromatography, can be followed by reverse-phase C4 HPLC	>95% purity 11400 µg L^{-1}	This publication

Biochemical characteristics of recombinant PF4

rhPF4 and platelet-derived PF4 had identical amino acid sequences when sequenced by LC-MS with the exception of an extra initial methionine in rhPF4, which is a characteristic of all recombinant proteins expressed in this bacterial system. When tested by MALDI-TOF, rhPF4 had a molecular weight of 7895.2 Da which is similar to the molecular weight of platelet-derived PF4 (7763.8 kDa) but is slightly larger due to the additional initiating methionine (monoisotopic mass of methionine = 131.04 Da) at the N-terminus. Cross-linking experiments and gel filtration chromatography were both performed to determine the oligomeric state of rhPF4 and its similarity to platelet-derived PF4. Treatment of rhPF4 and platelet-derived PF4 with BS^3 resulted in three cross-linked

products. The three bands correspond to the molecular weights of PF4 dimers, trimers, and tetramers formed, with both rhPF4 and platelet-derived PF4 showing the same band patterns with increasing BS^3 concentrations (Figure 2.3). Additionally, both platelet-derived PF4 and rhPF4 eluted from the Superdex 75 at a volume corresponding to the molecular weight of a tetrameric PF4 (approximately 31 kDa) based on a standard molecular weight ladder (Figure 2.4). Size exclusion chromatography showed that most of the rhPF4 is a tetramer with a small proportion of dimer and monomer species (Figure 2.4).
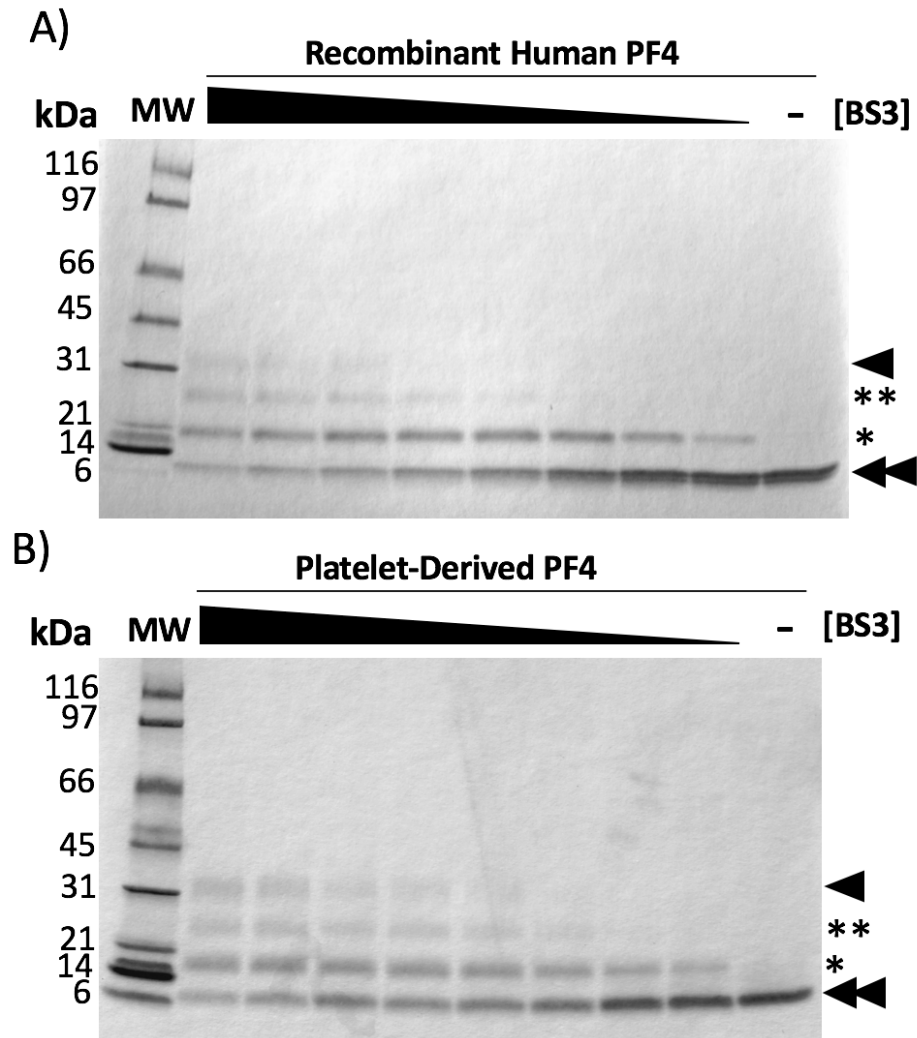
Figure 2.3: Oligomerization of rhPF4 and Platelet-derived PF4 through BS3 Cross-linking. The proteins were incubated in the presence/absence of BS3 and the reaction products were resolved by SDS-PAGE. From left to right, the gels show molecular weight markers (MW), A) rhPF4 and B) platelet-derived PF4 incubated with decreasing concentrations of BS3 (400 to 0 µM). Monomers (approx. 7.9 kDa) are indicated by a double arrowhead. Dimers (approx. 16 kDa) and trimers (approx. 24 kDa) are indicated with one or two asterisks, respectively. The presence of crosslinked products corresponding to the tetrameric PF4 (approx. 32 kDa) is indicated by an arrowhead.
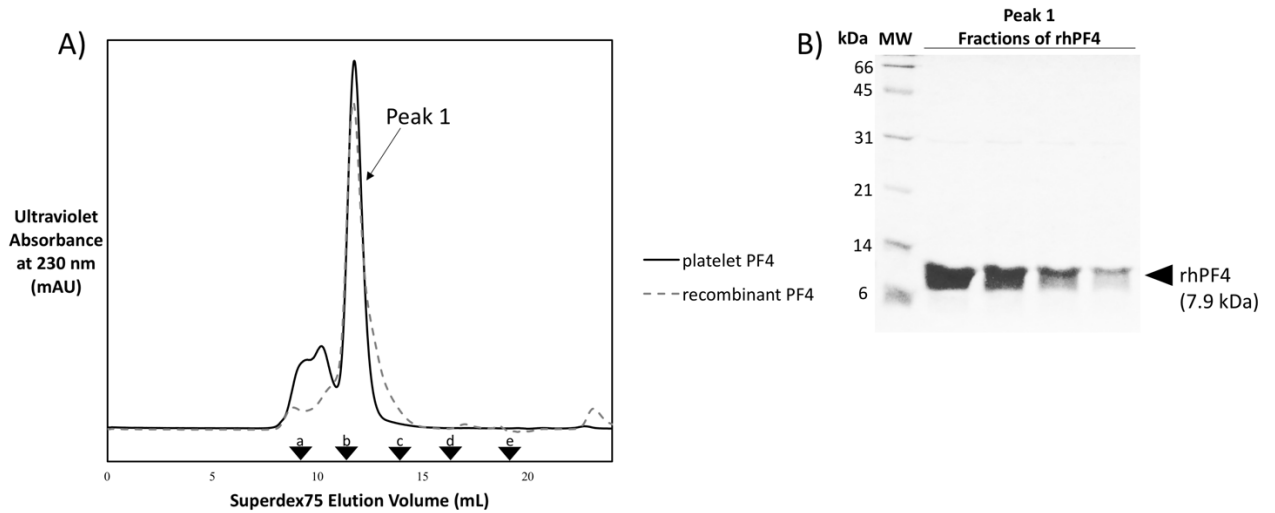
Figure 2.4: Comparing Elution Chromatograms of platelet-derived PF4 and rhPF4 using gel filtration chromatography. **A)** Platelet-derived PF4 and rhPF4 were analyzed on the Superdex75 to identify oligomeric states. Solid line represents the elution profile for platelet-derived PF4. Dashed lines represent the elution profile for rhPF4. 8 mL is the void volume of the Superdex75 and an elution volume of approximately 11 mL equates to 28 kDa. **B)** Purity of peak was confirmed on coomassie stained 18% SDS-polyacrylamide gel. rhPF4 in solution is predominantly tetrameric and the size is similar to that of platelet-derived PF4. Elution volumes for molecular weight standards used to calibrate the column are indicated with arrows: a) BSA (67 kDa); b) ovalbumin (43 kDa); c) ribonuclease A (13.7 kDa); d) aprotonin (6.5 kDa); e) vitamin B12 (1.4 kDa).

Antigenic and functional characteristics of recombinant PF4

Using sera from patients tested for HIT, we showed that the absorbance readings from the anti-PF4/heparin EIA with platelet-derived PF4 or rhPF4 were highly correlated ($r = 0.9545$, $n = 194$, $p < 0.0001$) (Figure 2.5). Of all serum samples tested in the anti-PF4/heparin EIA (n=194), 27 were SRA-positive. All 27 (100%) tested positive with both platelet-derived and rhPF4 in the anti-PF4/heparin EIA. In addition, rhPF4 produced similar serotonin release values ($r = 0.9597$, $n = 39$, $p < 0.0001$) compared with platelet-derived PF4 in the PF4-SRA. The PF4-SRA with rhPF4 correctly identified all 29 SRA-positive sera and excluded all 10 SRA-negative sera tested (Figure 2.6).
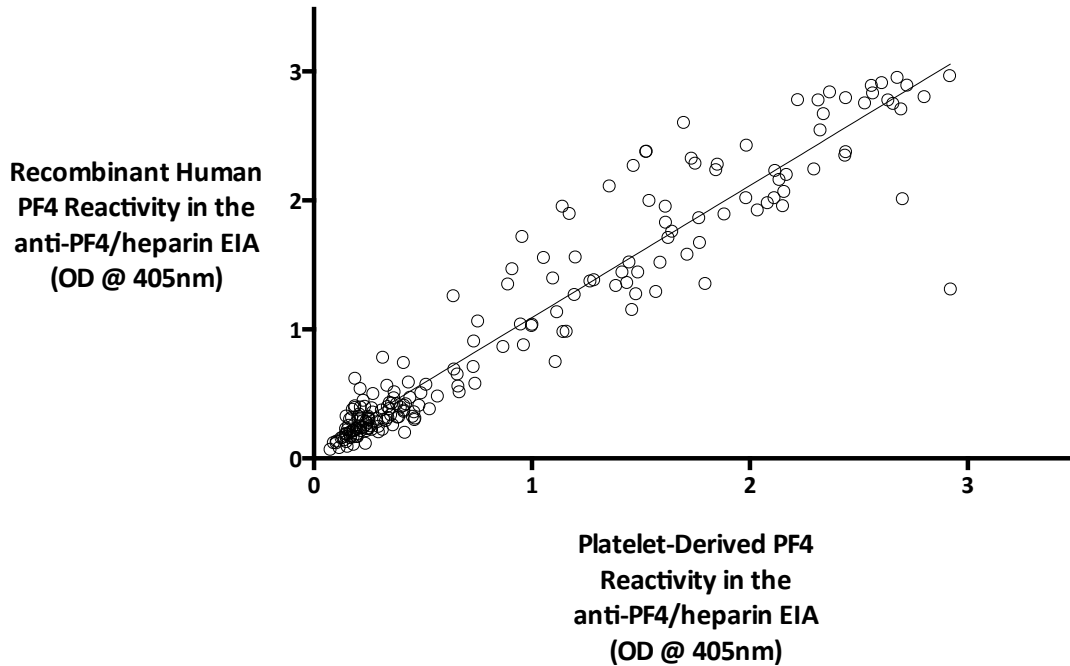
Figure 2.5: Comparing the Reactivity of Platelet-derived PF4 and rhPF4 in the anti-PF4/heparin EIA. Scatterplot comparing the results obtained (OD @ 405nm) from patient samples that include SRA-positive (EIA-positive and SRA-positive, n=27) or SRA-negative (EIA-positive and SRA-negative, n=54) and sera from healthy donors or patients that tested EIA-negative (n=113) in the anti-PF4/Heparin EIA using rhPF4 and platelet-derived PF4 absorbances at 405nm. Pearson's correlation coefficient was calculated (r = 0.9545). These results and the correlation demonstrate that there is comparable antigenicity and endpoint readings between platelet-derived PF4 and rhPF4. Of the 27 SRA-positive samples tested, rhPF4 was able to identify all sera similarly to that of platelet-derived PF4.

2.6 DISCUSSION

A sustainable source of PF4 is needed for biological, therapeutic, and diagnostic use. Newer diagnostic assays for HIT require exogenous PF4 (Padmanabhan et al., 2015b) as do commercial kits. In this report, we describe a novel technique that produces large amounts of pure rhPF4 with biochemical, antigenic and functional characteristics that mimic wild-type (platelet-derived) PF4.
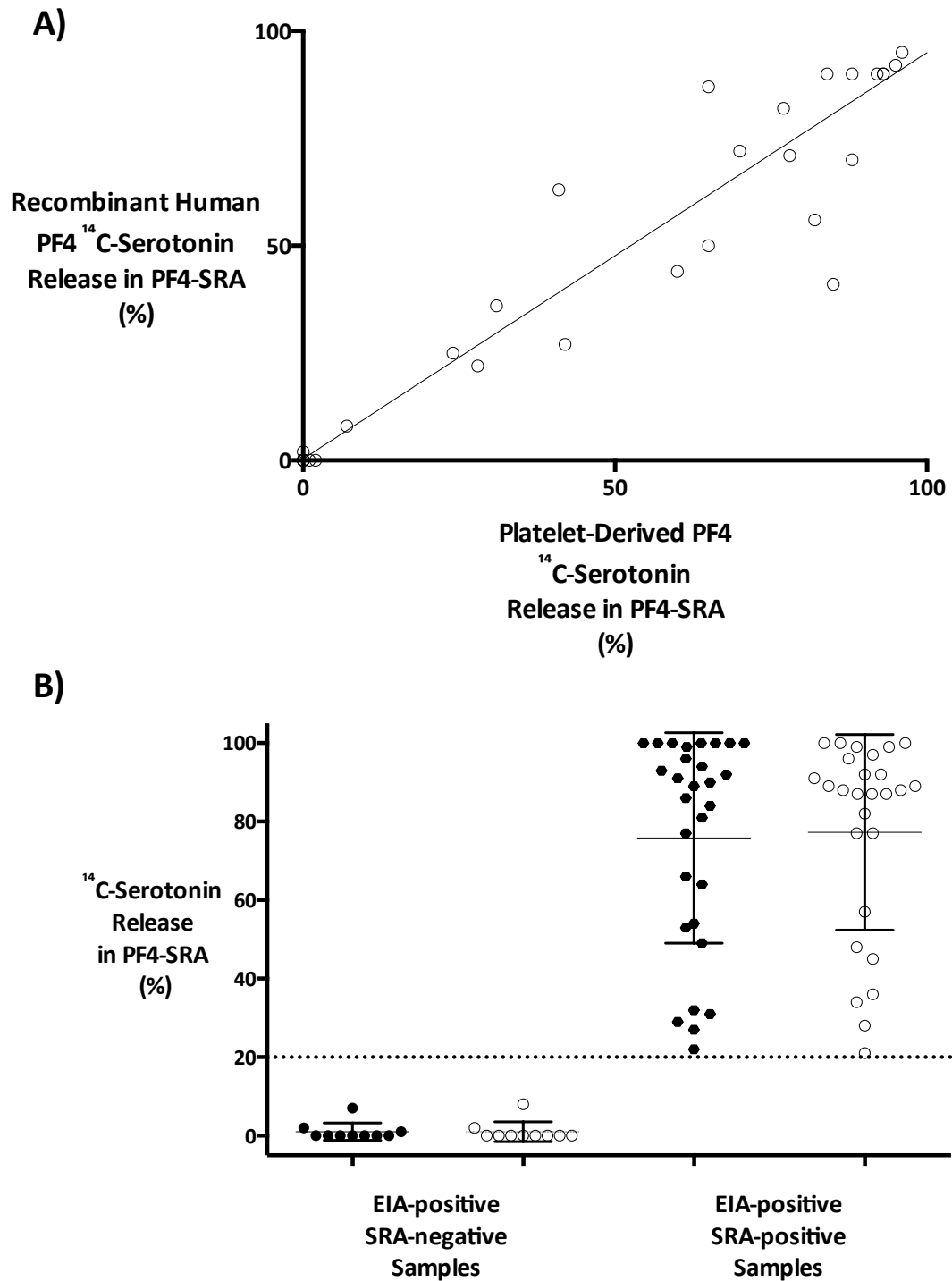
Figure 2.6: Comparing the Reactivity of Platelet-derived PF4 and rhPF4 in the PF4-SRA. A) Scatterplot comparing the results obtained (% release of ^{14}C-Serotonin) from 29 SRA-positive patient samples and 10 EIA-positive, SRA-negative patient samples in the

PF4-SRA at 50 µg mL^{-1} of rhPF4 or platelet-derived PF4. Pearson's correlation coefficient was calculated (r = 0.9597). These results and the correlation demonstrate that there is comparable antigenicity and endpoint readings between platelet-derived PF4 and rhPF4. **B)** Dot plot comparing the percent release of serotonin in the PF4-SRA with recombinant (black circles) or platelet-derived PF4 (open circles) as an exogenous source. Both recombinant and platelet-derived PF4 exhibit similar serotonin release within each category of patient sera. HIT negative samples were from suspected HIT patients who tested negative in the EIA. HIT positive samples were from suspected HIT patients who tested positive in both the EIA and SRA. Data for reactivity with 50 µg mL^{-1} PF4 in the PF4-SRA are shown.

Some key disadvantages of producing eukaryotic proteins in *E. coli* are the limited post-translational machinery (Khow and Suntrarachun, 2012) and the potential for low levels of soluble and functional recombinant proteins (Jana and Deb, 2005). This is demonstrated in previous methods which have been limited by low PF4 yields or low PF4 solubility. We overcame these limitations by increasing the expression levels of PF4 using a different plasmid vector designed for the overexpression of recombinant proteins in *E. coli*, optimizing the DNA sequence for codon bias within *E. coli*, and increasing solubility by adding specific detergents to the lysis process. Although glycosylation is present in other PF4 homologues, the glycosylation site on human PF4 is found in the amino-terminus which has high variability when compared to other mammalian species and is cleaved to form mature PF4 (Proudfoot AE, 1995)·(Ravanat et al., 1994). PF4 is approximately 7.8 kDa and undergoes minimal post-translational modifications (disulfide bonds)*(Hermodson et al., 1977).* Therefore, the production of rhPF4 in *E.coli* presents a rapid and efficient method for producing PF4 for laboratory or commercial use. The primary amino acid sequence of rhPF4 produced from this purification method is identical to the human PF4 except for an additional initiating methionine at the N-

terminus. *E. coli* normally express a methionine aminopeptidase that is able to remove the initiating methionine from expressed proteins with the subsequent amino acid being small and uncharged (Ben-Bassat et al., 1987). The methionine in the rhPF4 is not removed as a result of it being followed by a glutamic acid (Xiao et al., 2010). However, the presence of the methionine did not affect reactivity in the anti-PF4/heparin EIA or the PF4-SRA.

Producing recombinant proteins in prokaryotic systems has many advantages. *E. coli* has an unparalleled rate of growth (Sezonov et al., 2007), reagents are readily available and relatively inexpensive, the bacteria is easy to manipulate with mutagenesis, production-yields are usually high, and transformation with exogenous DNA is stable (Pope and Kent, 1996). Although, cost and time advantages depend on multiple components, overall, the described purification method encompasses both an efficient and high yield of rhPF4. One of the first described purification methods of PF4 from bacteria used a fusion moiety with additional amino acids at the N- and C-termini, use of cyanogen bromide cleavage, and guanidine extraction for large scale, due to insolubility of the protein (Myers et al., 1991). However, these additional amino acids are suboptimal for subsequent biochemical studies. Another purification method was developed using *E. coli* as the host but instead, the primary amino acid sequence of the rhPF4 was identical to hPF4, except for an initiating methionine residue (Park et al., 1990). Although simpler than the initial protocol, its yield was low, making it difficult to produce the large amounts required for structural and functional studies. Some key disadvantages of producing eukaryotic proteins in *E. coli* are the limited post-translational machinery

(Khow and Suntrarachun, 2012) and the potential for low levels of soluble and functional recombinant proteins (Jana and Deb, 2005).

Most recently, researchers used a method with an insect expression system to produce PF4 (Sachais et al., 2012). Insect expression systems are a higher eukaryotic system, therefore allowing for more complex post-translational modifications (Kollewe, 2013). The disadvantages of this system when compared to expression in *E. coli* is that it has higher costs, some of the post-translational modifications are not the exact same as in other eukaryotes, and it is a longer process (Brondyk, 2009).

Our novel method of producing rhPF4 has resulted in a practical source of PF4 for biochemical studies and for use in HIT antigenic and functional assays. When tested against platelet-derived PF4, rhPF4 identified all the SRA-positive samples, indicating that the PF4 produced is functionally comparable and suitable for use in HIT diagnostic assays. This source of rhPF4 will also facilitate studies targeting PF4 function in vivo and enable further studies of neoepitopes on this unique conformational antigen (Brandt et al., 2014).

2.7 ACKNOWLEDGEMENTS

Contribution: AH carried out the described studies, analyzed data, and wrote the manuscript. IN designed the research, interpreted data, and wrote the manuscript. JWS and JCM assisted with experimentation, provided technical assistance, and helped write the manuscript. DMA and JGK designed the research and helped write the manuscript. All authors reviewed and approved the final version of the manuscript.

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Chapter 3

Characterization of platelet factor 4 amino acids that bind pathogenic antibodies in heparin-induced thrombocytopenia

3.1 AUTHOR'S PREFACE

This chapter is focused on the understanding of pathogenic HIT antibodies we learned from the alanine scanning mutagenesis performed on PF4. Characterization of PF4 and HIT antibodies using alanine scanning mutagenesis revealed 5 amino acids on PF4 that affect the binding of pathogenic HIT antibodies. I developed a high-throughput method for producing 70 PF4 point mutations of PF4 to be used in the following EIAs (Figure 3.1). I performed, with the technical assistance of P. Horsewood, the EIAs with the 70 PF4 point mutations and different patient serum samples to determine which amino acids were important in their binding (Figure 3.2 and 3.3). I also expanded the amount of patient serum samples tested in the EIAs with potentially important PF4 mutants. J.W. Smith performed the PF4-SRAs to better characterize the patient serum sample population I was using in my EIAs. We found that KKO binding to PF4 depended on 13 amino acids, 3 of which are newly observed. We also found that 5 amino acids in PF4 may distinguish pathogenic from non-pathogenic antibodies. J.W. Smith and I analyzed the data and prepared the figures. Dr. I. Nazy, Dr. D.M. Arnold, Dr. J.G. Kelton, J.W. Smith and I wrote the manuscript.

3.2 ABSTRACT

Background: Heparin-induced thrombocytopenia (HIT) is an adverse drug reaction that results in thrombocytopenia and in some patients, thrombotic complications. HIT is mediated by antibodies that bind to complexes of platelet factor 4 (PF4) and heparin. The antigenic epitopes of these anti-PF4/heparin antibodies have not yet been precisely defined, because of the polyspecific immune response that characterizes HIT.

Objectives: To identify PF4 amino acids essential for binding pathogenic HIT antibodies.

Methods: Alanine scanning mutagenesis was utilized to produce 70 single point mutations of PF4. Each PF4 mutant was used in an EIA to test their capacity to bind a platelet-activating murine monoclonal anti-PF4/heparin antibody (KKO) and HIT patient sera (n=9).

Results and Conclusions: We identified 13 amino acids that were essential for binding KKO since they directly affected either the binding site or the antigenic conformation of PF4. We also identified 10 amino acids that were required for the binding of HIT patient sera and 5 of these amino acids were required for binding both KKO and the HIT patient sera. The 10 amino acids required for binding HIT sera were further tested to differentiate pathogenic HIT antibodies (platelet-activating, n=45) and non-pathogenic antibodies (EIA-positive, but non-platelet activating, n=28). We identified 5 mutations of PF4 that were recognized to be essential for binding pathogenic HIT antibodies. Using alanine scanning mutagenesis, we characterized possible binding sites of pathogenic HIT antibodies on PF4.

3.3 INTRODUCTION

Heparin is a widely-used anticoagulant and about 3-5% of patients can develop a severe drug reaction known as heparin-induced thrombocytopenia (HIT)(Martel et al., 2005, Kelton and Warkentin, 2008). HIT occurs when antibodies against multimolecular complexes of platelet factor 4 (PF4, CXCL4) and heparin activate platelets and monocytes (Amiral, 1992, Visentin et al., 1994, Greinacher, 1994, Kelton et al., 1994, Witt and Lander, 1994, Mikhailov et al., 1999, Dai et al., 2018). PF4 is a tetrameric chemokine with four cysteines, which form two disulfide bonds. PF4 undergoes a conformational change when bound to heparin, exposing neoepitopes that can induce the production of anti-PF4/heparin antibodies (Kreimann et al., 2014). The tetrameric structure of PF4 is critical in the formation of the ultralarge complexes that HIT antibodies recognize (Rauova et al., 2005). The PF4/heparin-antibody complex binds to the FcγRIIa on the platelet surface, as well as FcγRI on monocytes, cross-linking the receptors causing cellular activation and thrombin generation (Chong et al., 1989, Kelton et al., 1988, Pouplard et al., 2001, Arepally and Mayer, 2001, Reilly, 2001). The increased platelet activation in HIT leads to thrombocytopenia with an increased risk of thrombotic complications (Warkentin and Kelton, 1996). Many heparin treated patients produce anti-PF4/heparin antibodies, especially after cardiac or orthopedic surgery; (Amiral et al., 1995, Warkentin et al., 2000, Trossaert et al., 1998, Visentin et al., 1996) however, only a small subset of these patients will develop pathogenic anti-PF4/heparin antibodies that activate platelets and lead to HIT (Trossaert et al., 1998, Untch et al., 2002).

The antibody response in HIT patients is polyclonal and polyspecific, making it difficult to identify a common pathogenic epitope (Suh et al., 1998, Ziporen et al., 1998). Our group recently showed that approximately 36% of samples from suspected HIT patients that are positive in the enzyme immunoassay (EIA) but negative in the serotonin release assay (SRA), have low titers of platelet-activating anti-PF4/heparin antibodies, adding further complexity in determining the epitope specificity of HIT antibodies (Nazi et al., 2015b). We have defined non-pathogenic antibodies as EIA-positive samples which do not cause platelet activation, even in the presence of excess PF4 (Nazi et al., 2015b). Our previous work screening peptides of PF4 with patient sera showed that a majority of HIT antibodies recognize a noncontiguous conformational epitope on PF4 when it binds heparin (Horsewood et al., 1996). Other studies have defined two antibody binding sites on PF4 recognized by HIT patient serum antibodies: one site includes amino acids immediately C-terminal to the third cysteine of PF4 and the second includes the N-terminus and proline at position 34 near the third cysteine (Li et al., 2002, Ziporen et al., 1998). An X-ray crystallography study has shown KKO, a monoclonal anti-PF4/heparin antibody, binds to specific amino acids on three monomers of the PF4 tetramer (Cai et al., 2015). Although studies of KKO are useful in providing models for the pathogenesis of HIT, patient sera need to be studied in order to fully define the complexity of the immune response in HIT.

The disparities between anti-PF4/heparin antibodies that activate platelets (pathogenic HIT antibodies) and those that do not (non-pathogenic anti-PF4/heparin

antibodies) present a significant challenge in diagnosing HIT patients (Nazi et al., 2015a).
This is because of the absence of a rapid and widely available laboratory diagnostic test
with high specificity that can distinguish pathogenic from non-pathogenic antibodies
(Cuker, 2011, Warkentin et al., 1995). Depending upon the patient population, most of
these positive samples are clinically irrelevant and do not cause HIT (Lo et al., 2007,
Pouplard, 1999). Diagnostic accuracy for HIT is improved with the additional use of
functional HIT assays, such as the serotonin release assay (SRA) and the heparin-induced
platelet aggregation (HIPA) assay (Sheridan et al., 1986, Greinacher et al., 1991). These
tests are more specific than EIAs but are technically demanding and are available only in
a small number of specialized reference laboratories.

Studies have yet to determine the amino acids in PF4 that are integral in the
binding of anti-PF4/heparin antibodies found in HIT patients. More importantly,
identifying the amino acids that can separate pathogenic from non-pathogenic anti-
PF4/heparin antibodies has important implications for HIT testing. In this study, we used
alanine scanning mutagenesis to identify epitopes on PF4 that are essential for binding
KKO and pathogenic antibodies found in HIT patient sera.

3.4 MATERIALS AND METHODS

Patient samples and HIT antibody testing

KKO (ATCC, Manassas, VA, USA), a HIT-like murine monoclonal antibody specific for PF4/heparin complexes was initially screened to demonstrate whether alanine scanning mutagenesis can be used to describe possible epitopes on PF4 and to confirm previously reported sites. Serum samples used for these studies were obtained from patients referred for diagnostic testing to the McMaster Platelet Immunology Laboratory. We used patient sera that were divided into four groups: i) confirmed HIT sera (n=9), which were confirmed clinically with the 4Ts score and were EIA-positive/SRA-positive; ii) suspected HIT patient sera (EIA-positive/SRA-positive, n=45); iii) patients who did not have HIT but their sera had anti-PF4/heparin antibodies (EIA-positive/SRA-negative, n=28). Patients who did not have HIT but had anti-PF4/heparin antibodies were further divided into two subgroups: patient samples that tested positive in the PF4-SRA (EIA-positive/SRA-negative/PF4-SRA-positive), indicating they had subthreshold levels of pathogenic HIT antibodies, and patient samples that tested negative in the PF4-SRA (EIA-positive/SRA-negative/PF4-SRA-negative) indicating the presence of only non-pathogenic anti-PF4/heparin antibodies. Samples were tested using an in-house PF4/heparin IgG-specific enzyme immunoassay [EIA, optical density (OD) ≥ 0.45 was considered positive] and a heparin-dependent SRA ($\geq 20\%$ ^{14}C-serotonin release was considered positive).(Sheridan et al., 1986, Horsewood et al., 1996) All samples had test results for the EIA and SRA (Table S3.1)(Lo et al., 2006). These patient samples all had a

positive EIA OD_{405nm} ranging between 0.496-4.034. This study was approved by the Hamilton Integrated Research Ethics Board (HIREB).

Construction and expression of recombinant PF4 mutants

The full-length DNA coding sequence of human PF4 (Deuel, 1977) was cloned into the pET22b expression vector using restriction sites *Nde*I and *Hind*III (GenScript, Piscataway, NJ, USA). Mutants of PF4 were designed where non-alanine amino acids in wild-type PF4 were mutated to alanine and the alanine amino acids in wild-type PF4 were mutated to valine. PF4 mutants were introduced into *E. coli* ArcticExpress (DE3) cells (Agilent Technologies, Santa Clara, CA, USA. Overexpression of PF4 mutants cultures were grown at 37°C to mid-exponential phase, before adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and growing at 37°C for 3 hours. *E. coli* cells (20 mg wet weight) for each wild-type PF4 or PF4 mutant were lysed in 20 mM sodium phosphate, pH 7.2, 400 mM NaCl, 1.4 mM ß-mercaptoethanol, 5% (v/v) glycerol, 1% (v/v) Triton X-100 (Thermo Fisher Scientific, Waltham, MA, USA), and 0.5% (w/v) sodium deoxycholate (Sigma-Aldrich, St. Louis, MO, USA) with 2 mM $MgCl_2$, 10 µg/mL DNase I (Sigma-Aldrich) and an EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Protein expression was assessed for each PF4 mutant using 4-18% SDS polyacrylamide gels. Five PF4 mutants (C10A, C12A, C36A, I42A, and C52A) were purified as previously reported for wild-type PF4 and then analyzed on a Superdex 75 10/300 GL (GE Healthcare, Mississauga, ON, CAN) equilibrated with 1.5M NaCl/PBS

buffer.(Huynh et al., 2018) ImageJ (U.S. National Institutes of Health, Bethesda, MD, USA) was used to quantify the expression of each PF4 mutant from SDS-PAGE.

PF4/heparin IgG-specific enzyme immunoassay using streptavidin and biotinylated-heparin

The binding of anti-PF4/heparin antibodies to wild-type PF4 and PF4 mutants was measured using a modified PF4/heparin IgG-specific EIA (Horsewood et al., 1996)·(Stearns et al., 1997). 96-well NUNC Maxisorp plates (Thermo Fisher Scientific) were pre-coated with 10 µg/mL streptavidin and 1 IU/mL biotinylated-heparin and blocked with phosphate buffered saline (PBS) supplemented with 3% bovine serum albumin (BSA) for 2 hours at ambient temperature. Soluble cell lysate of PF4 or PF4 mutants was then added and incubated for 1 hour at ambient temperature. Two µg/mL KKO and 0.1 µg/mL rabbit polyclonal anti-PF4 (LifeSpan Biosciences Inc., Seattle, WA, USA) were also added to wells and incubated for 1 hour at ambient temperature. After washing, alkaline phosphatase-conjugated anti-mouse IgG or alkaline phosphatase-conjugated anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) was added at a 1:4000 dilution and incubated for 1 hour at ambient temperature. Addition of 1 mg/mL p-nitrophenylphosphate (PNPP, Sigma-Aldrich) substrate dissolved in 1 mol/L diethanolamine buffer (pH 9.6) was added for detection. Following a 30-minute incubation, the optical density was measured at 405nm (OD_{405nm}) using a TECAN Sunrise plate reader (Tecan Group Ltd., Mannedorf, Switzerland) to assess binding of antibodies to wild-type PF4 and PF4 mutants. Polyclonal rabbit anti-

PF4 was used to control and detect the amount of PF4 bound to the heparin in each well. Results were reported as a percentage of binding relative to wild-type PF4 binding using the following formula:

$$\% \text{ binding relative to WT} = \frac{\left(\text{Abs405}_{\text{mutant}}\big/\text{Abs405}_{\text{WT PF4}}\right)_{\text{Test antibody}}}{\left(\text{Abs405}_{\text{mutant}}\big/\text{Abs405}_{\text{WT PF4}}\right)_{\text{Polyclonal anti}-\text{PF4}}}$$

Variation in expression and solubility of the PF4 mutants was accounted for using the ratio of OD_{405nm} from the polyclonal rabbit anti-PF4. PF4 mutant amino acids that were deemed essential for binding to the test antibody when a greater than 75% reduction binding occurred in comparison to wild-type PF4 based on the calculation.

The method described above was also used to screen PF4 mutants binding to confirmed HIT sera, EIA-positive/SRA-positive and EIA-positive/SRA-negative patient sera at a 1:50 dilution. Binding was detected using alkaline phosphatase-conjugated anti-human IgG (Jackson Immuno Research Laboratories, Inc) as the secondary.

PF4-Serotonin release assay

Previously tested EIA-positive/SRA-negative patient samples (n=28) were tested in the PF4-SRA to determine the presence of low-titer platelet-activating antibodies (pathogenic antibodies)(Nazi et al., 2015b). Platelets were labelled with ^{14}C-serotonin and prepared as described for the standard SRA and incubated with test sera in the presence of exogenous PF4 (0, 50, and 100 μg/mL)(Kelton et al., 1988). After a 1 hour incubation with shaking, PBS-EDTA was added and the plates were centrifuged. An

aliquot of supernatant was removed from each well and added to scintillant and ^{14}C-serotonin release was measured using a scintillation counter (Packard Topcount, Meriden, CT, USA). KKO and serum from an SRA-positive patient were used as positive controls. The PF4-SRA was also used to determine if the selected PF4 mutants (C10A, C12A, C36A, I42A, and C52A) that had a significant decrease in binding between EIA-positive/SRA-positive and EIA-positive/SRA-negative sera can form the immune complexes capable of causing platelet activation using KKO and a confirmed HIT patient serum. Recombinant wild-type PF4 and PF4 mutants were produced as previously described (Huynh et al., 2018).

Statistical Analysis

The Mann-Whitney U test was used to compare 2 groups of either confirmed HIT samples or EIA-positive/SRA-positive patient samples with EIA-positive/SRA-negative samples. All other analyses used the unpaired Student's t-test and all data were analyzed using GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA, USA). A p value < 0.05 was considered statistically significant.

3.5 RESULTS

Expression and heparin-binding of wild-type PF4 and PF4 mutants

PF4 mutants were produced in *E. coli* and their expression was visualized using SDS-PAGE where relative expression was quantified compared to wild-type PF4. All

PF4 mutants expressed similarly to wild-type PF4 (mean = 99.2% compared to wild-type PF4, range = 91.5-104.4%). The ability of the PF4 mutants to also bind heparin was measured using an EIA-based streptavidin-biotinylated heparin capture system. The majority of PF4 mutants bound heparin similarly to wild-type PF4 (mean = 93.4%, range = 48-128%, Figure 3.1). Although single amino acids mutations were made in the known heparin binding region (amino acids R20A, R22A, H23A, T25A, K46A, R49A, K61A, K62A, K65A, K66A), no significant decrease in PF4 mutant binding to heparin was detected (Mayo et al., 1995, Cowan et al., 1986, Stuckey et al., 1992).
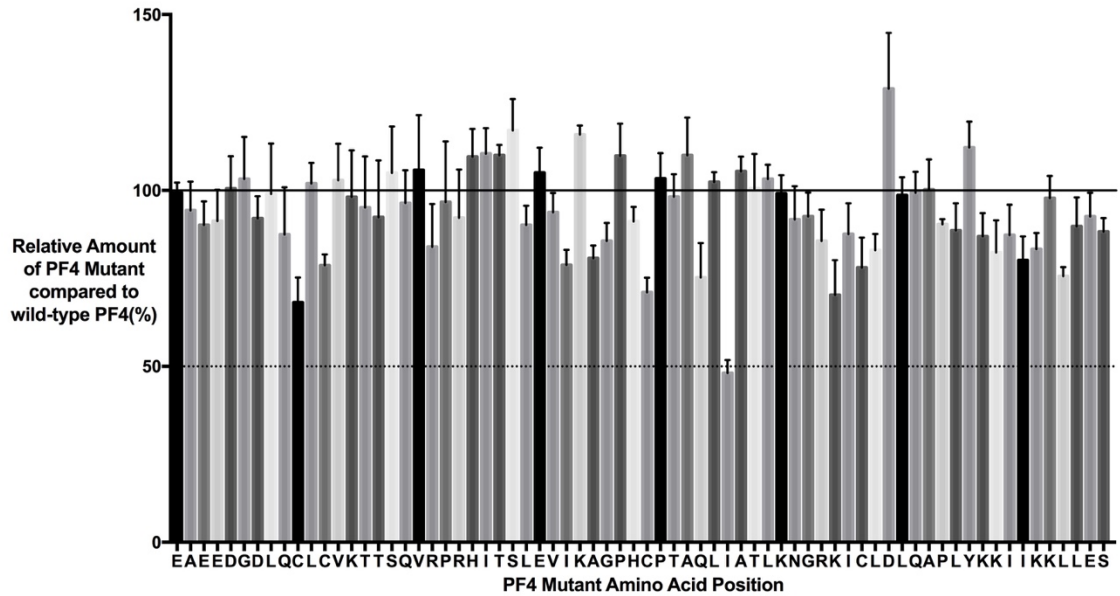
Figure 3.1: The relative heparin-binding to single point mutations of PF4 compared to wild-type PF4. The amount of mutant PF4 expressed and bound to heparin was determined using a streptavidin-biotinylated-heparin enzyme immunoassay capture system and detected using a rabbit polyclonal anti-PF4. Results are expressed as a percentage of the amount of heparin-bound wild-type PF4. Most mutants showed similar binding compared to wild-type PF4.

KKO binding to PF4 mutants

To determine if alanine scanning mutagenesis was able to identify PF4 amino acids important for antibody binding, we used KKO to confirm previously identified binding sites on PF4 (Cai et al., 2015, Li et al., 2002, Ziporen et al., 1998). Of the 70 PF4 mutants tested, thirteen PF4 mutants: L8A, Q9A, C10A, C12A, V13A, A32V, C36A, Q40A, I42A, C52A, L55A, L67A, and L68A, were identified with a 75% or greater reduction in KKO binding compared to wild-type PF4 (Table 3.1, Figure 3.2A). A 30-50% reduction in KKO binding was seen with PF4 mutants D7A, L11A, K15A, T16A, R20A, R22A, L27A, K31A, A43V, G48A, R49A, and L53A when compared to wild-type PF4. The remaining mutations of PF4 showed similar binding to KKO as wild-type PF4. All mutations of the cysteine amino acids of PF4 that abolished KKO binding were clustered close to other mutations that either abolished or reduced binding (Figure 3.2B). Conversely, eight mutations: A2V, E3A, E4A, D5A, Q18A, P58A, E69A, and S70A resulted in increased KKO binding (mean = 104.5%, range = 101-112%) when compared to wild-type PF4.
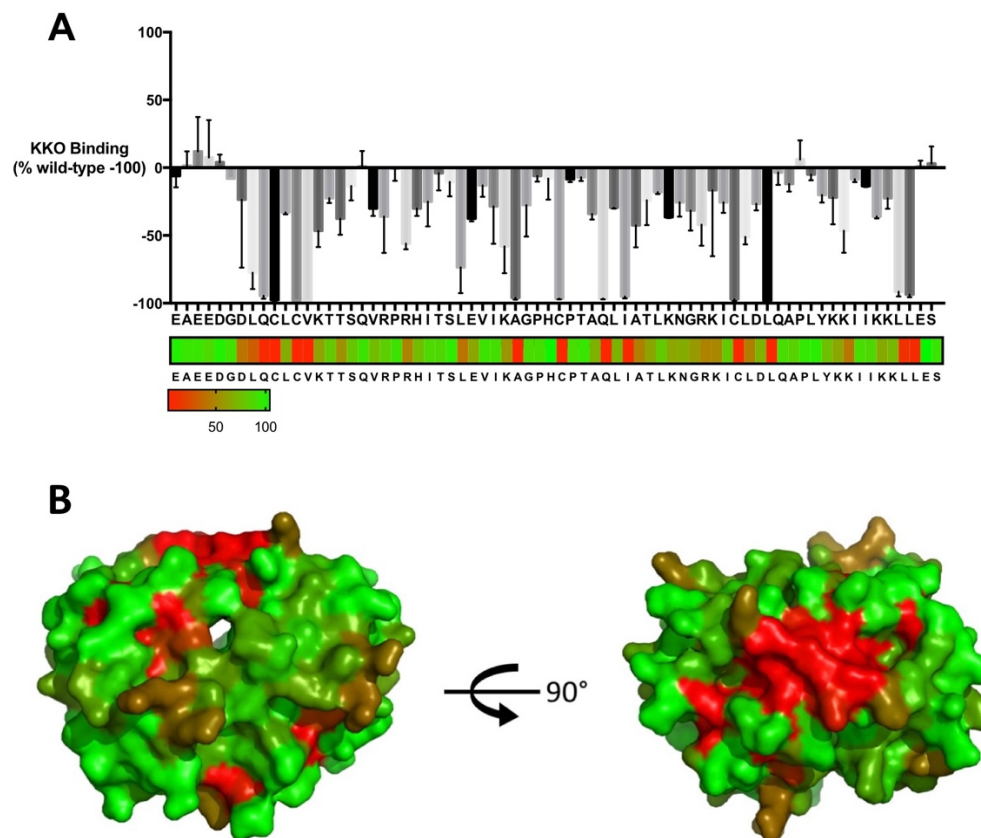
Figure 3.2: Binding of single point mutations of PF4 to KKO. A) The binding ability of KKO to each PF4 mutant relative to wild-type PF4 are shown. Negative values represent a loss of binding to KKO and positive values represent similar or increased binding to KKO relative to wild-type PF4 of n=2 experiments. Data reported as binding of PF4 mutant relative to wild-type PF4 and error bars correspond to the standard deviation. Data is also displayed as a heat map where KKO binding compared to wild-type PF4 is coloured using a red-green gradient, where red indicates that amino acid substitution resulted in a loss of binding and green indicates no change in binding. B) Surface representation of PF4 with binding data of PF4 mutants relative to wild-type PF4 imposed on 3D structure using the same red-green gradient as the heat map. Red indicates amino acid substitution that resulted in a loss of binding and green indicates no change in binding. Modified from Protein Data Bank entry 1RHP.

HIT sera binding to PF4 mutants

The interactions between PF4 mutants and anti-PF4/heparin antibodies were further characterized by testing all 70 PF4 mutants with confirmed HIT (n=9) and EIA-positive/SRA-negative (n=6) patient sera. The mean binding to each mutant was compared between these two groups. Ten PF4 mutants (Table 3.1) demonstrated a significant difference in reactivity between confirmed HIT and EIA-positive/SRA-negative sera (Figure 3.3). Five of the PF4 mutants that demonstrated a significant difference between confirmed HIT and EIA-positive/SRA-negative sera were among the 13 amino acids found important in the interaction with KKO (C10A, C12A, C36A, I42A, and C52A); whereas, the other 5 were found to only affect the binding of KKO (T15A, T38A, R49A, I51A, and D54A). Although, amino acid substitutions L8A, T16A, I30A, P37A, Q40A, K50A, L53A, and L67A reduced binding, the same trend was found in both the confirmed HIT and EIA-positive/SRA-negative sera (Figure 3.3). Similar to binding results for KKO, all cysteine to alanine mutations (C10A, C12A, C36A, C52A) resulted in a greater loss of binding with confirmed HIT sera when compared to EIA-positive/SRA-negative sera. PF4 mutants T15A, T38A, I42A, and D54A demonstrated normal binding to KKO but showed reduced binding to HIT sera (Figure 3.3).
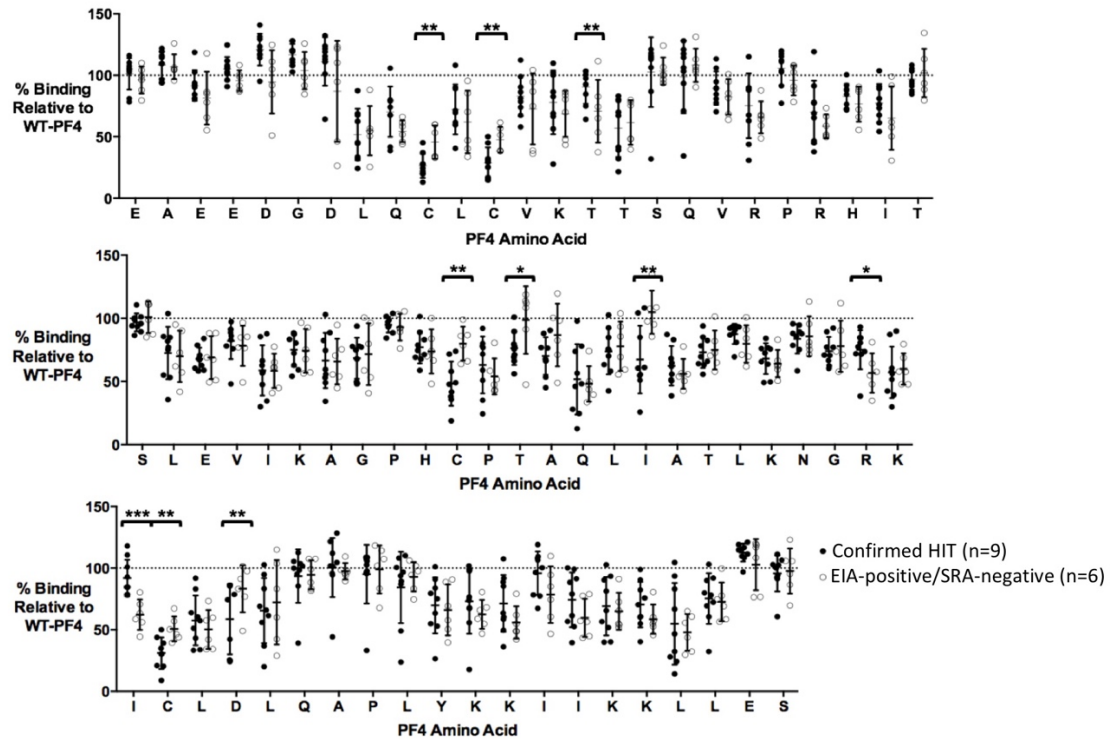
Figure 3.3: Binding of patient sera to single point mutations of PF4. Confirmed HIT (n=9) and EIA-positive/SRA-negative (n=6) patient sera were tested against all 70 single point mutations of PF4. The binding of patient sera to each PF4 mutant is shown as a percentage of binding relative to wild-type PF4. Data reported as mean PF4 mutant binding and error bars correspond to the standard deviation. The black closed circles represent the binding of confirmed HIT patient sera and the black open circles represent EIA-positive/SRA-negative patient sera. Student's t-test was performed to determine significant differences between the two antibody groups. Ten mutations (C10A, C12A, T15A, C36A, T38A, I42A, R49A, I51A, C52A and D54A) were determined to have a significant difference in binding between the confirmed HIT and EIA-positive/SRA-negative patient sera. Dotted line represents 100% binding or similar binding as wild-type PF4. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

We further investigated the importance of the 10 PF4 mutants of interest, as they showed the largest separation between pathogenic HIT antibodies and non-pathogenic anti-PF4/heparin antibodies, using a stepwise approach to better distinguish EIA-

positive/SRA-positive (containing pathogenic HIT antibodies) and EIA-positive/SRA-negative (containing non-pathogenic anti-PF4/heparin antibodies) patient sera. The 10 amino acid substitutions that had a significant difference (C10A, C12A, T15A, C36A, T38A, I42A, R49A, I51A, C52A and D54A) from the previous screen were tested against additional larger subset of samples from EIA-positive/SRA-positive (suspected HIT, n=45) and EIA-positive/SRA-negative (serologically does not have HIT, n=28) sera. The average binding to each mutant was compared between the two groups. Only the point mutations to the cysteines (C10A: 84.95% vs. 113.1%, $p<0.01$, C12A: 79.57% vs. 106.2%, $p<0.05$, C36A: 68.69% vs. 98.51%, $p<0.01$, C52A: 62.23% vs. 85.15%, $p<0.05$) and I42A (99.04% vs. 147.6%, $p<0.0005$) distinguished pathogenic samples from non-pathogenic samples (Figure 3.4A). The two mutants, R49A and I51A, that initially bound more to confirmed HIT sera than EIA-positive/SRA-negative (likely containing non-pathogenic anti-PF4/heparin) sera from the previous screen, no longer showed a significant difference when the number of sera was expanded (Figure 3.4A).
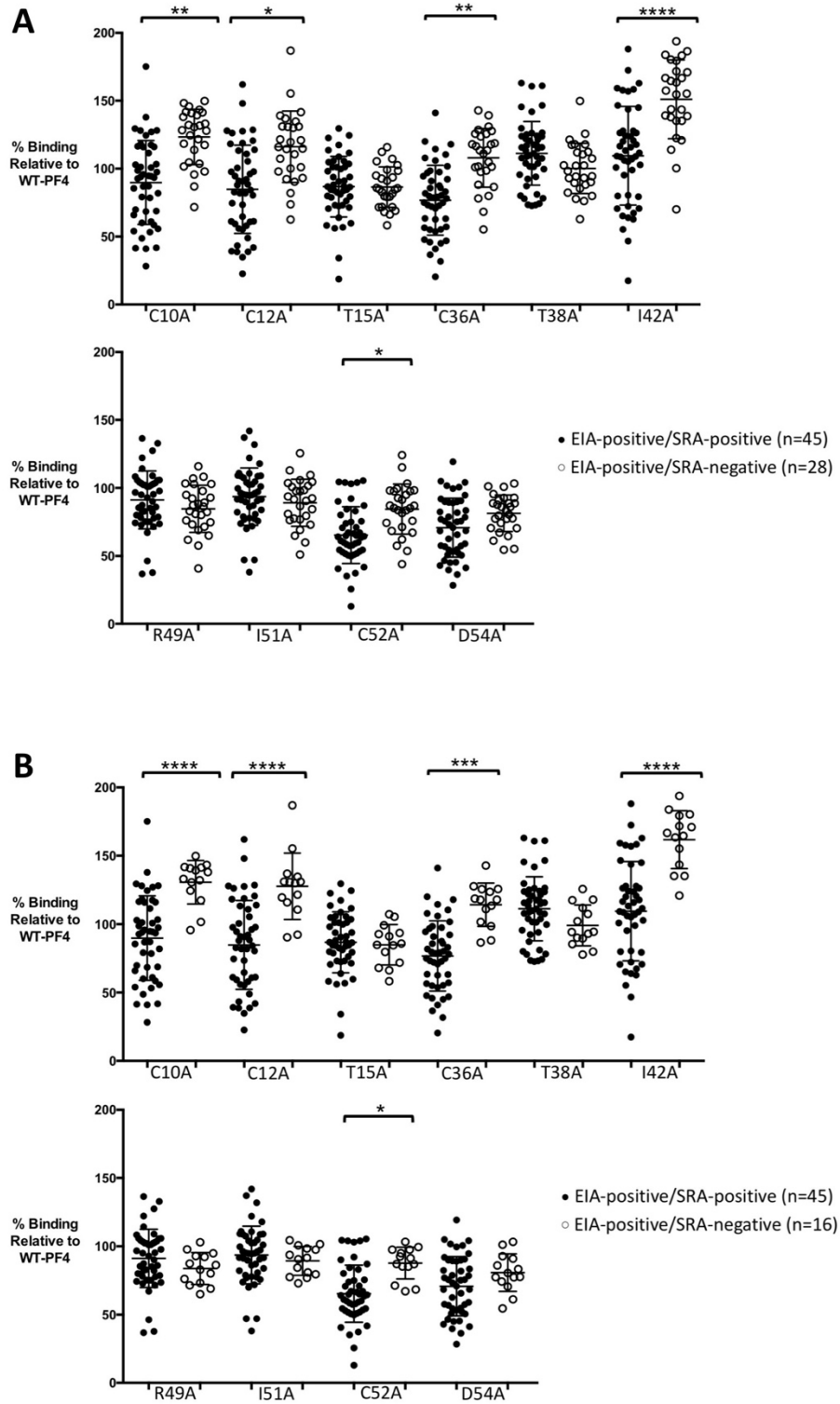
Figure 3.4: Selected PF4 mutants with the potential to separate pathogenic HIT antibodies and non-pathogenic anti-PF4/heparin antibodies. A) The 10 mutants that were found in the first alanine scanning mutagenesis screen (Figure 3.3) to have a significant difference in binding between the two groups were screened using additional EIA-positive/SRA-positive (n=45) and EIA-positive/SRA-negative (n=28) patient sera. The relative binding of the patient sera to selected PF4 mutants are shown as a percentage relative to wild-type PF4. B) 10 mutants that were found in the first alanine scanning mutagenesis screen (Figure 3.3) to have a significant difference in binding between the two groups were once again screened using EIA-positive/SRA-positive (n=45) and EIA-positive/SRA-negative (n=16) patient samples after 12 EIA-positive/SRA-negative samples that had reactivity in the PF4-SRA were removed. Data reported as mean PF4 mutant binding relative to wild-type PF4 and error bars correspond to the standard deviation. The black closed circles represent the binding of EIA-positive/SRA-positive patient sera and the black open circles represent EIA-positive/SRA-negative patient sera. Student's t-test was performed to determine significant differences between the two antibody groups. Of the 10 PF4 mutants that were initially deemed important to separate pathogenic from non-pathogenic antibodies, only 5: C10A, C12A, C36A, I42A, and C52A remained statistically significantly different between the two groups. * $p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0005$.

In an effort to further identify PF4 mutants that could better separate between pathogenic and non-pathogenic anti-PF4/heparin antibodies, we characterized patient sera by testing their ability to induce platelet activation and identify samples containing subthreshold levels of pathogenic HIT antibodies using the PF4-SRA (Nazi et al., 2015b). Of the EIA-positive/SRA-negative samples, 4/28 (14.3%) tested positive ($\geq$ 20% ^{14}C-serotonin release) and 8/24 (33.3%) demonstrated some release (>0% to <20% ^{14}C-serotonin release) in the PF4-SRA using 50 and 100 µg/mL of exogenous PF4. Thus, EIA-positive/SRA-negative samples with subthreshold levels of platelet-activating antibodies (positive in the PF4-SRA) were excluded from analysis to remove patient sera that could reduce the separation of antibody binding between EIA-positive/SRA-positive

and EIA-positive/SRA-negative patient sera. Binding of pathogenic HIT antibodies to the C10A ($89.82 \pm 30.86\%$ vs. $130.7 \pm 15.88\%$, $p<0.0005$), C12A ($84.86 \pm 32.49\%$ vs. $127.6 \pm 24.22\%$, $p<0.0005$), and C36A ($76.78 \pm 25.76\%$ vs. $114.2 \pm 15.8\%$, $p<0.001$) mutants increased in significance when compared to EIA-positive/SRA-negative patient sera that were negative in the PF4-SRA (Figure 3.4B).

PF4 mutants in the functional PF4-SRA

Since PF4 mutants C10A, C12A, C36A, and C52A were the most important in separating between pathogenic and non-pathogenic anti-PF4/heparin antibodies in the binding assays after strict analysis, they were tested for their ability to activate platelets, to confirm their role as part of the pathogenic epitope. Neither the C10A nor the C12A mutants supported platelet activation by confirmed HIT sera (Figure 3.5A). In contrast, C36A and I42A was able to stimulate a positive result ($\geq 20\%$ ^{14}C-serotonin release) in the PF4-SRA at both 50 and 100 µg/mL of the mutant (Figure 3.5A). Similarly, we also tested these PF4 mutants using KKO and found it did not induce a positive PF4-SRA result in the presence of C10A, C12A and C52A using 50 and 100 µg/mL of PF4 (Figure 3.5B). However, C36A and I42A was able to induce a positive serotonin release at 50 and 100 µg/mL in the PF4-SRA with KKO.

A

B
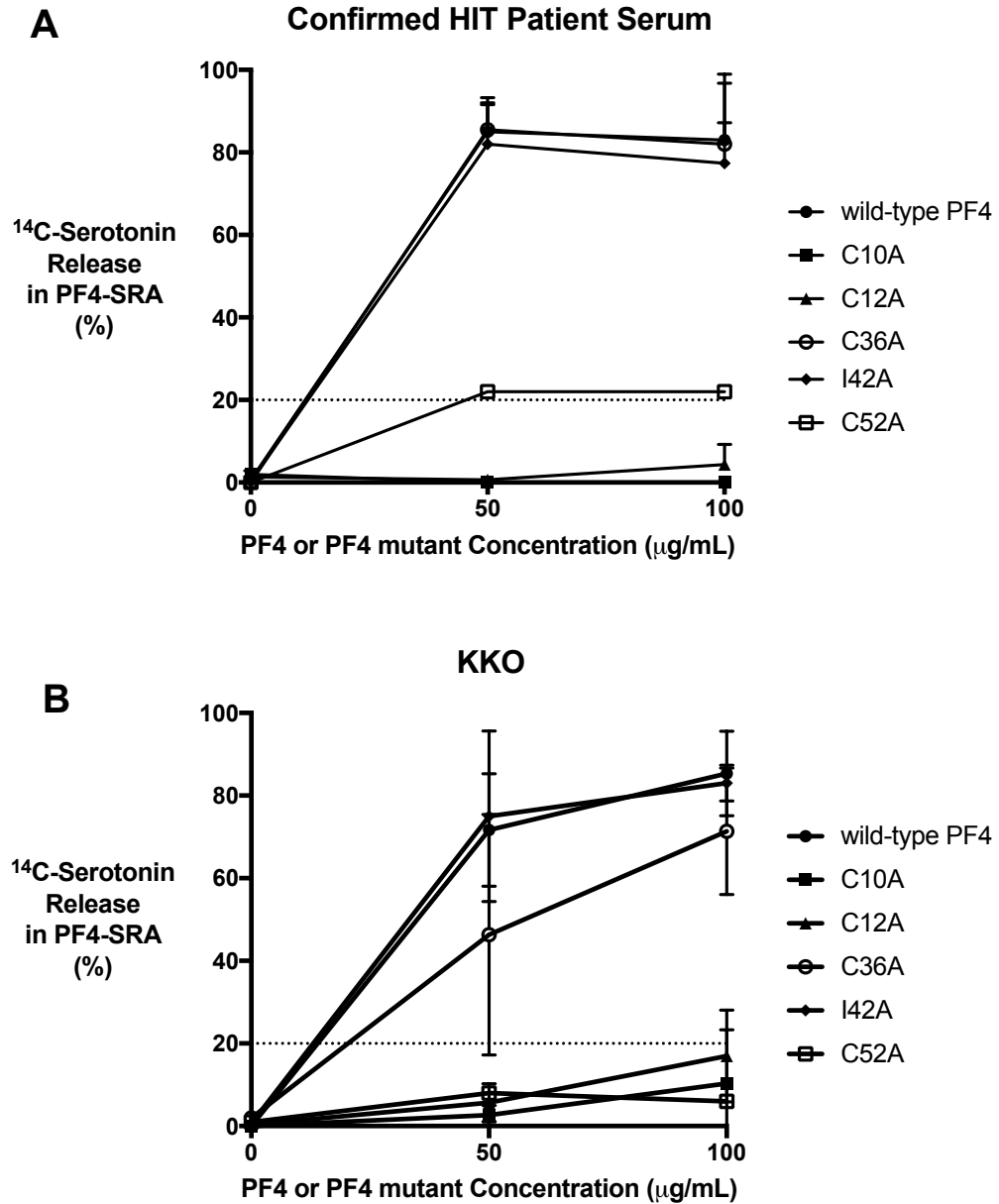
Figure 3.5: A confirmed HIT patient sample and KKO induced platelet activation using wild-type PF4 and selected PF4 mutants. The ability of PF4 mutants C10A, C12A, Q40A, and I42A to mediate ^{14}C-serotonin release in the presence of an activating anti-PF4/heparin patient serum or KKO, was measured using the PF4-SRA and compared to wild-type PF4 (wtPF4). These PF4 mutants were selected because they caused a binding reduction with KKO or with confirmed HIT samples. A ^{14}C-serotonin release of ≥ 20% is considered positive. I42A caused a similar release when compared to wild-type

PF4. PF4 mutants C10A, C12A, and Q40A did not result in a positive release. Data reported as mean serotonin release and error bars correspond to the standard deviation. The line with circles represents serotonin release mediated by wild-type PF4. The line with squares represents release for PF4 mutant C10A. The line with upright triangles represents serotonin release for PF4 mutant C12A. The line with inverted triangles represents serotonin release for PF4 mutant Q40A. The line with diamonds represents serotonin release for PF4 mutant I42A.

3.6 DISCUSSION

HIT is a potentially fatal thrombotic disorder mediated by antibodies to complexes that form between PF4 and heparin or cellular glycosaminoglycans (GAGs) (Kelton and Warkentin, 2008, Arepally and Ortel, 2006, Warkentin and Kelton, 1996). The precise epitopes of pathogenic HIT antibodies are currently unknown. In this report, we describe the use of alanine scanning mutagenesis to identify the binding sites of: (a) a murine HIT-like monoclonal antibody (KKO); (b) EIA-positive/SRA-positive sera; and (c) EIA-positive/SRA-negative sera with or without subthreshold levels of platelet-activating antibodies. Collectively, our study provides further insight into the PF4 amino acids important for binding pathogenic HIT antibodies.

Our results support previous work showing pathogenic HIT antibodies have a similar epitope on PF4 to that of KKO (Cai et al., 2015). We identified 13 amino acids in PF4 to be important for KKO binding. Our results agree with previous studies showing 10 amino acids, Q9, C10, C12, V13, A32, C36, Q40, C52A, L55, and L68 to be are part of the KKO epitope on PF4 (Ziporen et al., 1998, Li et al., 2002, Cai et al., 2015). In

71

addition, our data also implicate amino acids L8, I42, and L67 to be essential in the

binding of KKO to PF4. Although the side chain of L67 is more than 5Å away in the

crystal structure of PF4 and the KKO-Fab, L67 could still be considered an important

amino acid for the interaction with pathogenic HIT antibodies since this structure was not

crystallized with heparin, which has been shown to cause additional conformational

changes to the PF4 tetramer (Kreimann et al., 2014). The majority of amino acids

determined integral in the binding of KKO to PF4 can be found exposed on the surface of

the PF4 tetramer (Table 3.1). Surface amino acids designated as important by alanine

scanning mutagenesis likely contribute to the epitope required for KKO binding,

especially if they all localize to the same site on the X-ray crystal structure. However,

some amino acids were reported as buried in the PF4 tetramer. These amino acids may

still play a role in epitope for KKO as the surface and buried designations are from the X-

ray crystal structure without heparin (Park et al., 1990). A previous mutagenesis study

identified amino acids D7-Q9 as antigenic sites on PF4 for KKO (Cai et al., 2015).

Another study found amino acids in the region between the 3rd and 4th cysteines (C36 and

C52) to be important in KKO binding (Li et al., 2002). Our study confirmed these

findings since many PF4 amino acids within this region have reduced or abolished

binding when mutated. P34 and P37 have been previously shown to be important amino

acids for KKO and pathogenic HIT antibodies; (Ziporen et al., 1998) however, when both

prolines were mutated in our assay, they did not reduce binding of KKO to PF4

significantly. Our results may differ as we changed the proline amino acids to alanine

instead of arginine or asparagine. Depending on the location in the structure, there may

not be enough space to fit an arginine or asparagine and these mutations may have destabilized the structure and the binding indirectly. An alanine mutation avoids loss of binding associated with steric hindrance.

Some studies have shown that the majority of HIT pathogenic antibodies share a similar binding site to PF4 as KKO; however, others indicate up to 10% of the HIT pathogenic antibodies are thought to have a different binding site (Cuker et al., 2013, Sachais et al., 2012, Kizlik-Masson et al., 2017). Although probable HIT pathogenic epitopes were identified using KKO, HIT patient sera is required to identify the full spectrum of pathogenic HIT epitopes. Our alanine scanning mutagenesis results using patient sera showed a larger range of reactivity within the confirmed HIT and false-positive HIT groups. In this study, the screen using confirmed HIT and EIA-positive/SRA-negative patient antibodies identified 10 PF4 mutants (C10A, C12A, T15A, C36A, T38A, I42A, R49A, I51A, C52A and D54A) that had a significant difference in binding from wild-type PF4. Of these, 5 PF4 mutants (C10A, C12A, C36A, I42A, and C52A) showed a significant difference between EIA-positive/SRA-positive (suspected HIT with pathogenic HIT antibodies) and EIA-positive/SRA-negative (patient sera containing non-pathogenic anti-PF4/heparin antibodies) sera when screened using referred patient samples. C10A and C12A can both be found exposed on the surface of the PF4 tetramer and are likely a part of the pathogenic HIT epitope that binds the antibodies. Although different amino acids of PF4 were important for binding KKO and defined HIT patient sera, similar amino acids were also identified. This may be due to

HIT patient sera having anti-PF4/heparin antibodies that share an epitope with KKO, but there may be another epitope. EIA-positive/SRA-negative antibodies exhibited greater than 75% reduction in binding to very few amino acid substitutions of PF4. This is to be expected since EIA-positive/SRA-negative patient sera have predominantly non-pathogenic antibodies which are polyclonal, making it difficult to define a specific epitope for these antibodies.

The polyspecific immune response in HIT patients further complicates analysis (Nazi et al., 2015b, Ziporen et al., 1998, Suh et al., 1998, Li et al., 2002) and considerable overlap was observed between confirmed HIT (EIA-positive/SRA-positive) and false-positive (EIA-positive/SRA-negative) patient samples. The PF4-SRA was performed to identify low titers of pathogenic antibodies within EIA-positive/SRA-negative sera, identifying sera that exclusively contain non-pathogenic antibodies for further study. Although, statistical significance increased, no new amino acids could be identified as part of the pathogenic HIT epitope. This suggests that EIA-positive/SRA-positive and confirmed HIT samples also contain non-pathogenic anti-PF4/heparin antibodies and explains the difficulty in defining specific pathogenic HIT epitopes in our system. The tetramerization of PF4 is integral in pathogenic HIT antibody binding (Rauova et al., 2005). Changing the cysteines of PF4 disrupts the disulfide bonds and likely alters the structure, which may explain the loss of binding in both the EIA and the PF4-SRA. However, PF4 mutant C36A was able to induce platelet activation in the PF4-SRA but mutant C10A did not, despite being its counterpart in the disulfide bond. Analysis of the

X-ray crystal structure shows that C10 and C12 are exposed on the surface of PF4 and C36 is buried (Table 3.1). These results suggest that C10 and C12 are likely a part of the pathogenic HIT epitope and the loss of binding in the EIA for C36A is a result of a change in the structure and not due to a loss of direct binding to the antibodies.

Alanine scanning mutagenesis is a widely used high-throughput technique in determining the functional role of protein amino acids (Cunningham and Wells, 1989, Wells, 1991, Matthews, 1996). However, there are disadvantages to alanine scanning mutagenesis. Because we are using single mutations, we may miss cooperative amino acid interactions that may play a role in the epitope. In addition, it may be difficult to identify which amino acids make up the true epitopes found in HIT patient sera as the polyclonal nature of anti-PF4/heparin antibodies also found in patient sera may increase the background signal in our assay. Although, mutations were made to the heparin-binding site of PF4, it has been shown that PF4 can bind to heparin with the removal of some positively charged amino acids (Mayo et al., 1995). Mutating every amino acid of PF4 without discrimination could structurally change PF4, which can affect the binding indirectly, and not necessarily an indication of an amino acid that is part of the epitope for the binding of pathogenic HIT antibodies. Thus, our study identifies amino acids of PF4 that are likely part of the epitope of KKO and pathogenic HIT antibodies and these amino acids may be used to separate pathogenic HIT antibodies from the non-pathogenic anti-PF4/heparin antibodies. Additional studies are needed to further distinguish specific amino acids that are a part of the epitope of pathogenic HIT antibodies while reducing the

effect of polyclonal non-pathogenic antibodies. Further characterization of the ability of PF4 mutants to induce platelet activation can help define the amino acids that constitute the pathogenic epitope of HIT antibodies.

This study investigated pathogenic HIT antibodies within well-defined patient samples and suggests possible new PF4 amino acids as part of the epitope of pathogenic antibodies in HIT. Alanine scanning mutagenesis of PF4 has narrowed the epitope of pathogenic HIT antibodies to specific amino acids and better described the polyclonal nature of anti-PF4/heparin antibodies found in sera of suspected HIT patients. In addition, our studies reveal a group of patient samples that contain subthreshold levels of pathogenic HIT antibodies that although are present, are not sufficient to lead to HIT. Ultimately, these findings will help lead to a better understanding of the differences between pathogenic and non-pathogenic HIT antibodies and aid in the development of new tests for HIT with an increased diagnostic accuracy.

3.7 ACKNOWLEDGEMENTS

3.8 AUTHORSHIP CONTRIBUTIONS

Contribution: AH carried out the described studies, analyzed data, and wrote the manuscript. IN designed the research, interpreted data, and wrote the manuscript. JWS and PH performed experiments, provided technical assistance, and wrote the manuscript. RC and AG wrote and edited the final manuscript. DMA and JGK designed the research and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

3.9 DISCLOSURE OF CONFLICTS OF INTEREST

A. Huynh, D. M. Arnold, I. Nazy and J. G. Kelton have a patent on 'Mutant Platelet Factor 4 and Uses Thereof' pending. The other authors state that they have no conflict of interests.

Table 3.1: Binding activity of PF4 mutants to KKO, HIT-positive and HIT-negative patient sera.

PF4 Mutant	Binding to KKO (n=2)[a]	Area on protein[b]
Wild-type	100	-
L8A	22	Exposed
Q9A	5	Exposed
C10A	3	Exposed
C12A	2	Exposed
V13A	2	Exposed
A32V	4	Exposed/Buried
C36A	3	Buried
Q40A	3	Buried
I42A	5	Buried
C52A	3	Buried
L55A	2	Exposed
L67A	8	Buried
L68A	7	Buried

PF4 Mutant	Binding to confirmed HIT patient sera (n=9)[a]	Binding to EIA-positive/SRA-negative patient sera (n=6)[a]	Significant difference?[c]	Area on protein[b]
Wild-type	100	100		-
C10A	26.44±9.66	45.67±13.40	**	Exposed
C12A	28.78±12.56	47.50±10.63	**	Exposed
T15A	90.56±13.53	71.00±25.49	**	Exposed
C36A	48.44±17.58	80.00±13.43	**	Buried
T38A	76.33±13.29	98.50±26.99	*	Exposed
I42A	67.44±26.57	94.83±16.94	**	Buried
R49A	75.22±15.62	56.83±15.46	**	Exposed
I51A	92.00 ±14.91	62.17±12.45	***	Buried

C52A	30.89±12.83	50.50±10.09	**	Buried
D54A	58.56±28.54	83.33±19.19	**	Exposed

[a] % relative to wild-type PF4 binding at 100%

[b] Determined using relative solvent accessibility generated by PyMOL using a 25% cut-off

[c] * $p<0.05$, **$p<0.01$, ***$p<0.001$

Table S3.1: Test Results of the Confirmed HITs (n=9)

Patient #	SRA 0 U/mL[a]	SRA 0.1 U/mL[a]	SRA 0.3 U/mL[a]	SRA 100 U/mL[a]	Anti-PF4/heparin IgG EIA OD_{405nm}	4Ts Score
1	89	95	94	2	3.248	6
2	39	95	97	2	2.595	6
3	96	100	10	0	2.867	7
4	6	93	93	0	2.544	>4
5	99	100	100	1	2.737	8
6	45	100	99	1	2.68	5
7	97	97	96	0	2.891	6
8	78	100	99	1	3.029	8
9	98	99	100	1	2.794	7

[a] % release of serotonin

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Chapter 4

Pathogenic antibodies in heparin-induced thrombocytopenia specifically target an immunodominant region on platelet factor 4

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4.1 AUTHOR'S PREFACE

This chapter focusses on further defining the necessary components of pathogenic HIT antibodies that lead to platelet activation. Our initial data with alanine scanning mutagenesis showed that most antibodies bind to a specific area on PF4. If all anti-PF4/heparin antibodies bind to a certain area on PF4, does then the affinity of the antibody correlate to its ability to activate platelets and lead to clinical HIT was our next question. I developed a high-throughput method for producing 70 PF4 point mutations of PF4 to be used in the following EIAs (Figure 4.1). I performed, with the technical assistance of P. Horsewood, the EIAs with the 70 PF4 point mutations and different monoclonal antibodies to determine which amino acids were important in their binding. R. Clare and M. Ivanova performed the BLI experiments on the same monoclonal antibodies and characterized patient sera. R. Clare and I analyzed the data and prepared the figures. Dr. I. Nazy, Dr. D.M. Arnold, Dr. J.G. Kelton, R. Clare and I wrote the manuscript.

4.2 ABSTRACT

Introduction: Heparin induced thrombocytopenia (HIT) is an adverse drug reaction that occurs when heparin binds to platelet factor 4 (PF4) forming immunogenic multimolecular complexes. Anti-PF4/heparin IgG antibodies then bind PF4/heparin complexes, leading to cross-linking of FcγIIa receptors on platelets and FcγRI on monocytes, resulting in platelet activation, thrombocytopenia and possible thrombosis. Antibody class (IgG-specific) and titre (OD > 1) are necessary but not sufficient determinants of pathogenic anti-PF4/heparin antibodies. The objective of this study was to identify the other biochemical and structural determinants that are characteristic of pathogenic HIT antibodies. We compared antibody specificity and affinity of pathogenic HIT antibodies compared with non-pathogenic anti-PF4/heparin antibodies.

Methods: In this study, we used sera with anti-PF4/heparin antibodies from patients with confirmed HIT (n=10) and patients post-cardiopulmonary bypass (CPB; n=10). Results were compared with healthy controls (n=10). Confirmed HIT patients met clinical criteria for HIT and tested positive in the anti-PF4 IgG/A/M immunoassay and in the serotonin release assay (SRA). CPB patients had all received heparin, tested positive in the immunoassay (OD > 0.4; range 0.42 - 2.73) and tested negative in the SRA. We determined antibody specificity using a panel of PF4 proteins with sequentially mutated amino acids and in-silico structural analysis. We determined affinity using biolayer interferometry (BLI). KKO, a murine monoclonal HIT-like antibody, was used as a control.

Results: When 30 PF4 mutants were used to test the effect of the amino acid changes on the binding of HIT and CPB patient sera, an average of 8 different PF4 amino acids per confirmed HIT sera resulted in more than 35% loss of binding when compared to wild-type PF4 whereas none of the 30 PF4 mutants resulted in more than 35% loss of binding to CPB sera. Structural analysis demonstrated that the amino acids of PF4 that significantly affected the binding of HIT sera, but not CPB sera, were clustered to a specific region on PF4, similar to the region of KKO. Of the two categories of patient sera and healthy controls tested using BLI, the PF4/heparin antibodies of confirmed HIT patients had the strongest binding (0.8592 ± 0.2802 response units, $p<0.01$) when compared to CPB (-0.02010 ± 0.02980 response units) and healthy controls (-0.2146 ± 0.02758 response units).

Conclusion: This work shows that among the polyclonal response in HIT, antibodies have to bind to the pathogenic HIT region with high affinity to ensure the proper spatial configuration for Fc-receptor cross-linking that results in platelet activation and subsequently HIT. This work is important in that it will serve as the basis for future diagnostic and therapeutic approaches for HIT.

4.3 INTRODUCTION

Heparin remains the standard of care in clinical settings both for prophylactic and therapeutic anticoagulation; however, in 1-3% of patients who receive heparin, it is paradoxically capable of causing severe thrombotic complications (Warkentin et al., 1995). Heparin-induced thrombocytopenia (HIT) is an adverse drug reaction that occurs when heparin binds to the self-protein, platelet factor 4 (PF4) and forms immunogenic multimolecular complexes. PF4 undergoes a conformational change when bound to heparin, exposing neoepitopes that can induce the production of anti-PF4/heparin antibodies.(Kreimann et al., 2014) The tetrameric structure of PF4 is critical in the formation of the ultra-large complexes that HIT antibodies recognize.(Rauova et al., 2005) As a result, anti-PF4/heparin IgG antibodies are formed, which bind to these complexes, cross-linking Fc receptors on platelets and monocytes causing intense platelet activation, thrombocytopenia and thrombosis (Kelton and Warkentin, 2008). The increased platelet activation in HIT leads to thrombocytopenia with an increased risk of thrombotic complications.(Warkentin and Kelton, 1996). Many heparin treated patients produce anti-PF4/heparin antibodies, especially after cardiac or orthopedic surgery (Amiral et al., 1995, Warkentin et al., 2000, Trossaert et al., 1998, Visentin et al., 1996); however, only a small subset of these patients will develop pathogenic anti-PF4/heparin antibodies that activate platelets and lead to HIT (Trossaert et al., 1998, Untch et al., 2002).

The antibody response in HIT patients is polyclonal and polyspecific, making it difficult to identify a common pathogenic epitope.(Suh et al., 1998, Ziporen et al., 1998) Other studies have defined two antibody binding sites on PF4 recognized by HIT patient serum antibodies: one site includes amino acids immediately C-terminal to the third cysteine of PF4 and the second includes the N-terminus and proline at position 34 near the third cysteine.(Li et al., 2002, Ziporen et al., 1998) An X-ray crystallography study has shown KKO, a monoclonal anti-PF4/heparin antibody, binds to specific amino acids on three monomers of the PF4 tetramer.(Cai et al., 2015) Previous studies on anti-PF4/heparin antibodies in patient sera have shown that the majority of HIT pathogenic antibodies share a similar binding site to PF4 as to a murine monoclonal antibody against human PF4/heparin known as KKO; however, others indicate up to 10% of the HIT pathogenic antibodies are thought to have a different binding site (Cuker et al., 2013, Sachais et al., 2012, Kizlik-Masson et al., 2017). Specifically, KKO binds PF4/heparin complexes that causes thrombocytopenia and thrombosis in a transgenic mouse model (Arepally, 2000). KKO is used as a model pathogenic HIT antibody for antigenicity studies as it has been shown to compete with pathogenic HIT antibodies for binding to PF4 *in vitro* and form the ultra large immune complexes associated with HIT (Cuker et al., 2013). Sachais et al. reported that KKO is able to cluster PF4 tetramers using precipitation of complexes of radiolabeled PF4 and KKO. However, Nguyen et al. did not observe binding of KKO to PF4 alone in fluid-phase. Recently, 5B9, an anti-PF4/heparin monoclonal antibody, with a human Fc was developed that fully mimics the effects of

human HIT antibodies. Therefore, 5B9 binds epitopes on PF4 that are likely critical for pathogenic HIT antibodies (Kizlik-Masson et al., 2017).

The disparities between anti-PF4/heparin antibodies that activate platelets (pathogenic HIT antibodies) and those that do not (non-pathogenic anti-PF4/heparin antibodies) present a significant challenge in diagnosing HIT patients (Nazi et al., 2015a). Though the presence of anti-PF4/heparin antibodies in serum is a compulsory component for HIT manifestation, the antibodies are heterogeneous in their specificity and affinity for the antigen. A study by Amiral et al. (2000) investigated plasma from different patients and found that anti-PF4/heparin antibodies within one sample had different reactivities. They also saw that the higher affinity antibodies induced the strongest platelet activation in the SRA (Amiral et al., 2000). These results are similar to recent work by Nguyen et al. (2017), who also found that anti-PF4/polyanion antibodies bind with different strengths to PF4/heparin complexes. Different binding strengths of antibodies to their antigen can be either due to differences in their recognition of the antigen or the ability of the antibody to bind with one or both Fab arms (Nguyen et al., 2017).

Additionally, both studies determined that there exists different classes of antibodies found within a patient serum: anti-PF4/heparin antibodies with low binding strengths that do not activate platelets, anti-PF4/heparin antibodies with sufficient binding

strengths that can activate platelets, and a third class of heparin-independent HIT antibodies that are able to bind the PF4 tetramers and make the epitope accessible for anti-PF4/heparin antibodies that can cause platelet activation. Antibodies obtained from human sera are typically polyclonal and this is reflected by the ability to find all these antibody groups from one sample. These observations support the statement that while anti-PF4/heparin antibodies are found in 25-50% of all heparin-exposed patients, the incidence of clinical HIT is much lower. It also suggests that affinity differences may contribute to variations in pathogenicity.

Little is known about the features of the antigen-binding part (Fab) of anti-PF4/heparin antibodies, which determine their biological effects. More importantly, is there a specific epitope on PF4 for pathogenic HIT antibodies that the polyclonal pool of antibodies found in patient serum must contain to lead to HIT. In this study, we propose that characteristics an anti-PF4/heparin antibody requires to cause subsequent platelet activation and clinical HIT bind to a specific area on PF4 and have a strong binding capacity for PF4/heparin complexes.

4.4 MATERIALS AND METHODS

Patient samples and HIT antibody testing

Monoclonal antibodies KKO (ATCC, Manassas, VA, USA) and 5B9 were used in alanine scanning mutagenesis and biolayer interferometry (BLI). Participants included

healthy volunteers who denied previous heparin exposure (n=10), patients exposed to heparin during cardiac surgery with cardiopulmonary bypass (CPB, n=10) and patients diagnosed with HIT (n=10). Serum from post-surgery CPB patients were collected, since up to 70% of CPB patients produce anti-PF4/heparin antibodies (Selleng et al., 2010b, Warkentin and Greinacher, 2003). HIT diagnosis was confirmed using the 4Ts score where all HIT patients had a clinical score of 4 and 9/10 (90%) experienced thrombosis, as well as a positive commercially available PF4 enhanced heparin-dependent IgG/A/M-specific enzyme immunoassay (EIA, Immucor, WI, USA) [optical density (OD) $\geq$ 0.4 and a positive heparin-dependent serotonin-release assay (SRA) ($\geq$ 20% ^{14}C-serotonin release)(Sheridan et al., 1986). Serum from HIT patients were collected at a median 14.3 days (range: 6-27) after heparin administration. Median age of healthy controls (n=10) was 45.0 years (range: 25.4 – 61.9) and 6 (60.0%) were female; median age of HIT patients (n=10) was 69.6 years (range: 52.8 – 89.0) and 5 (50.0%) were female (Table 4.1). Samples were heat-inactivated at 56°C for 30 minutes and centrifuged for 10 minutes at 300 x g, after which the supernatant was collected and diluted 1/32 and tested in duplicate. This study was approved by the Hamilton Integrated Research Ethics Board (HIREB) and informed written consent was obtained from all participants.

Table 4.1 Characteristics of healthy controls, post-surgery cardiopulmonary bypass (CPB) heparin-treated patients and HIT patients.

	Healthy controls (n=10)	CPB patients (n=10)	HIT patients (n=10)
Sex, n (F/M)	6/4	2/8	5/5

Heparin administration, n (%)	0 (0%)	10 (100%)	10 (100%)
4Ts Score ≥ 4, n (%)	-	-	10 (100%)
Thrombosis, n (%)	-	-	9 (90%)
PF4/heparin IgG/A/M EIA (OD ≥ 0.4), n (%)	0 (0%)	10 (100%)	10 (100%)
Positive SRA (% release ≥ 20), n (%)	-	0 (0%)	10 (100%)

None of the CPB patients developed HIT; however, all (100%) seroconverted [Optical Density (OD) ≥ 0.4] as tested by the PF4/heparin-dependent IgG/A/M enzyme immunoassay (EIA) and none tested positive in the serotonin-release assay (SRA) (≥ 20% ^{14}C-serotonin release). All HIT patients included in this study were diagnosed using the 4Ts score (≥4), a positive PF4/heparin-dependent IgG/A/M-specific EIA and positive serotonin-release assay (SRA).

Construction and expression of recombinant PF4 mutants

The full-length DNA coding sequence of human PF4 (Deuel, 1977) was cloned into the pET22b expression vector using restriction sites *Nde*I and *Hind*III (GenScript, Piscataway, NJ, USA). Thirty mutants of PF4 were expressed and purified as previously described (Huynh et al., 2018, Huynh et al., 2019). These thirty amino acids of PF4 were selected for this study based on their loss of reactivity with KKO and 5B9 when mutated. Briefly, mutants of PF4 were designed where non-alanine amino acids in wild-type PF4 were mutated to alanine and the alanine amino acids in wild-type PF4 were mutated to valine. PF4 mutants were introduced into *E. coli* ArcticExpress (DE3) cells (Agilent Technologies, Santa Clara, CA, USA. Overexpression of PF4 mutants cultures were grown at 37°C to mid-exponential phase, before adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and growing at 37°C for 3 hours. *E. coli* cells for each wild-type PF4 or PF4 mutant were lysed in 20 mM sodium phosphate, pH 7.2, 400 mM NaCl, 1.4 mM ß-mercaptoethanol, 5% (v/v) glycerol, 1% (v/v) Triton X-100 (Thermo

Fisher Scientific, Waltham, MA, USA), and 0.5% (w/v) sodium deoxycholate (Sigma-Aldrich, St. Louis, MO, USA) with 2 mM $MgCl_2$, 10 µg/mL DNase I (Sigma-Aldrich) and an EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Protein expression was assessed for each PF4 mutant using 4-18% SDS-polyacrylamide gels.

PF4/heparin IgG-specific enzyme immunoassay using streptavidin and biotinylated-heparin

Measuring the effect of the 30 amino acids on the binding of anti-PF4/heparin antibodies in patient sera was analyzed like previously described (Huynh et al., 2019). The binding of anti-PF4/heparin antibodies to wild-type PF4 and PF4 mutants was measured using a modified PF4/heparin IgG-specific EIA (Horsewood et al., 1996)·(Stearns et al., 1997). 96-well NUNC Maxisorp plates (Thermo Fisher Scientific) were pre-coated with 10 µg/mL streptavidin and 1 IU/mL biotinylated-heparin and blocked with phosphate buffered saline (PBS) supplemented with 3% bovine serum albumin (BSA) for 2 hours at ambient temperature. Soluble cell lysate of PF4 or PF4 mutants was then added and incubated for 1 hour at ambient temperature. Two µg/mL KKO, two µg/mL 5B9 and 0.1 µg/mL rabbit polyclonal anti-PF4 (LifeSpan Biosciences Inc., Seattle, WA, USA) were also added to wells and incubated for 1 hour at ambient temperature. After washing, alkaline phosphatase-conjugated anti-mouse IgG or alkaline phosphatase-conjugated anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) was added at a 1:4000 dilution and incubated for 1 hour at ambient temperature. Addition of 1 mg/mL p-nitrophenylphosphate (PNPP, Sigma-

Aldrich) substrate dissolved in 1 mol/L diethanolamine buffer (pH 9.6) was added for

detection. Following a 30-minute incubation, the optical density was measured at 405nm

(OD_{405nm}) using a TECAN Sunrise plate reader (Tecan Group Ltd., Mannedorf,

Switzerland) to assess binding of antibodies to wild-type PF4 and PF4 mutants.

Polyclonal rabbit anti-PF4 was used to control and detect the amount of PF4 bound to the

heparin in each well. Results were reported as a percentage of binding relative to wild-

type PF4 binding using the following formula:

$$\% \text{ binding relative to WT} = \frac{\left(\text{Abs405}_{\text{mutant}}/\text{Abs405}_{\text{WT PF4}}\right)_{\text{Test antibody}}}{\left(\text{Abs405}_{\text{mutant}}/\text{Abs405}_{\text{WT PF4}}\right)_{\text{Polyclonal anti-PF4}}}$$

Variation in expression and solubility of the PF4 mutants was accounted for using the

ratio of OD_{405nm} from the polyclonal rabbit anti-PF4. PF4 mutant amino acids that were

deemed essential for binding to the test antibody when a greater than 75% reduction

binding occurred in comparison to wild-type PF4 based on the calculation.

The method described above was also used to screen PF4 mutants binding to HIT

sera (n=9) and CPB patient sera (n=3) at a 1:50 dilution. Binding was detected using

alkaline phosphatase-conjugated anti-human IgG (Jackson Immuno Research

Laboratories, Inc) as the secondary.

Labelling PF4 with biotin

Wild-type PF4 and PF4 mutants were incubated with 5 times the volume of

Heparin Sepharose 6 Fast Flow (GE Healthcare) for 1 hour with shaking at ambient

temperature. EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) was added to the PF4 and heparin sepharose mixture in 20 molar excess and allowed to react for 1 hour with shaking at ambient temperature. Assuming total biotinylation, the now biotinylated wild-type PF4 or PF4 mutants were eluted from the heparin sepharose using PBS and 2M NaCl. Absorbance at 280 nm was measured using a spectrophotometer (Eppendorf) and used to calculate the concentration.

Levels of biotinylation of PF4 was checked using an EIA where 10 μg/mL of streptavidin in bicarbonate buffer (pH 9.6) was coated onto microtiter plates and incubated overnight at 4˚C. The wells were washed with PBS+0.05% Tween 20 and PBS and then blocked with 3% BSA in PBS for 2 hours at ambient temperature. 100 μL/well of 60 μg/mL biotinylated PF4 diluted in 1% BSA was then added to the wells and incubated for 1 hour at ambient temperature. 0.1 μg/mL polyclonal rabbit anti-PF4 (LifeSpan Biosciences Inc.) diluted in 1% BSA were added to the wells and incubated for 1 hour at ambient temperature. After washing, 100 μL/well 1:5000 alkaline phosphatase-conjugated affiniPure goat anti-rabbit IgG, Fcγ Fragment Specific (Jackson Immuno Research Laboratories, Inc.) diluted in 1% BSA were added to the wells and incubated for 1 hour at ambient temperature. Wells were washed and followed by the addition of 1 mg/mL PNPP substrate dissolved in 1 mol/L diethanolamine buffer (pH 9.6). Following a 30-minute incubation, the optical density was measured at 405nm (OD_{405nm}) using a TECAN Sunrise plate reader to assess biotinylation levels of wild-type PF4.

Biolayer interferometry (BLI) using KKO, 5B9 and patient serum

BLI experiments were performed using the Octet-QK Red 96 (FortéBio, Menlo Park, CA, USA). Samples or buffer were dispensed into 96-well black flat-bottom microtiter plates (Greiner Bio-one, Kremsmünster, Austria) at a volume of 200 μL per well with an operating temperature maintained at 30°C. Streptavidin-coated biosensor tips (FortéBio, Menlo Park, CA, USA) were pre-wetted with phosphate buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO, USA) buffer in order to establish a baseline prior to antigen immobilization. Biotinylated recombinant PF4 (final concentration 7.5 μg/mL in PBS with 1% BSA) alone or complexed with 0.125 U/mL unfractionated heparin (LEO Pharma, Ballerup, Denmark) or with 1 μg/mL fondaparinux (GlaxoSmithKline, Brentford, UK) or 0.03125 U/mL enoxaparin (Sanofi, Paris, France) was then immobilized on the biosensor tips while agitating for 15 minutes at 1000 rpm. A baseline was then established by dipping the tips in PBS with 1% BSA for 13 minutes. Antigen-coated sensors were then submerged into antibody solution: 2 μg/mL KKO or 5B9 diluted in PBS with 1% BSA while agitating for 10 minutes at 1000 rpm. Sensors then moved on to dissociation step-wells containing buffer while agitating for 56 minutes at 1000 rpm. The method described above was also used to screen patient sera at a 1/32 dilution during the association step and each sample was tested in duplicate. Two negative controls were included in the assay, 200 μL buffer in place of both antigen and analyte and experimental concentration of antigen with 200 μL buffer in place of analyte only. Similar to KKO, each experiment was done twice,

once with biotinylated-PF4 as the antigen and once with biotinylated-PF4/heparin as the antigen.

Data acquisition and statistical analysis

Data were generated automatically by the supplied computer software (Octet® User Software version 3.1) and analyzed using the 1:2 bivalent analyte binding model. Reference values from control wells were subtracted and all results were aligned to measured baseline. The binding profile response of each sample was summarized as a "nm shift" (the wavelength/spectral shift in nanometers), which represented the difference between the start and end of the association step. Correlation analysis were performed using the unpaired t-test with GraphPad Prism version (GraphPad Software, La Jolla, CA, USA). A p-value of < 0.05 was considered to be statistically significant.

4.5 RESULTS

KKO binding to PF4 mutants

Twelve PF4 mutants of the 30 PF4 mutants tested: L8A, Q9A, C10A, C12A, V13A, A32V, C36A, Q40A, C52A, L55A, L67A, and L68A, were identified with a 75% or greater reduction in KKO binding compared to wild-type PF4. A 50-75% reduction in KKO binding was seen with PF4 mutants D7A, L11A, K15A, T16A, R20A, R22A, L27A, K31A, A43V, G48A, R49A, and L53A when compared to wild-type PF4. The remaining mutations of PF4 showed similar binding to KKO as wild-type PF4. All

mutations of the cysteine amino acids of PF4 that abolished KKO binding were clustered close to other mutations that either abolished or reduced binding.

5B9 binding to PF4 mutants

Of the 30 PF4 mutants tested, ten PF4 mutants: L8A, C10A, C12A, T15A, T16A, A32V, Q40A, C52A, and L53A were identified with a 75% or greater reduction in 5B9 binding compared to wild-type PF4. A 50-75% reduction in 5B9 binding was seen with PF4 mutants Q9A, K14A, R22A, and P37A when compared to wild-type PF4. The remaining mutations of PF4 showed similar binding to 5B9 as wild-type PF4. Conversely, three mutations: A39V, L41A, and L55A resulted in increased 5B9 binding (mean = 112.9%, range = 102.8-129.9%) when compared to wild-type PF4. When the changes in binding are superimposed upon the structure of PF4, an area is highlighted, likely containing the epitope of 5B9 (Figure 4.1).

Patient sera binding to PF4 mutants

Nine HIT sera and three CPB sera were tested with the 30 PF4 mutants believed to form region on PF4 where all pathogenic HIT antibodies bind. No single amino acid of the 30 PF4 mutants tested within the defined region of PF4 affected all 9 HIT sera. Each HIT sera had a different combination of the 30 PF4 mutants that reduced binding. PF4 mutants C52A, L53A, D54A, and L67A reduced binding >35% with the majority of HIT sera tested (6, 7, 7, and 6 of the 9 HIT sera respectively). If the criteria is increased to a

reduction in binding >50% with PF4 mutants, the PF4 mutants that affect multiple HIT sera are L8A, C10A, C12A, C52A, L53A, D54A, and L67A.

Six of the HIT sera tested were affected (>35% loss of binding) by 6-14 PF4 mutants. Three of the HIT sera were only affected by 1-2 PF4 mutants when tested. However, although there was less of an effect, they still show similar amino acids impacted, just with less of a reduction in binding. Three PF4 mutants of the 30 tested did not reduce binding >35% with the nine HIT sera when compared to wild-type PF4: G6A, T15A, and S70A. When the 30 PF4 mutants were tested against the three CPB sera, none of the PF4 mutants caused a loss of binding >35% compared to wild-type PF4.
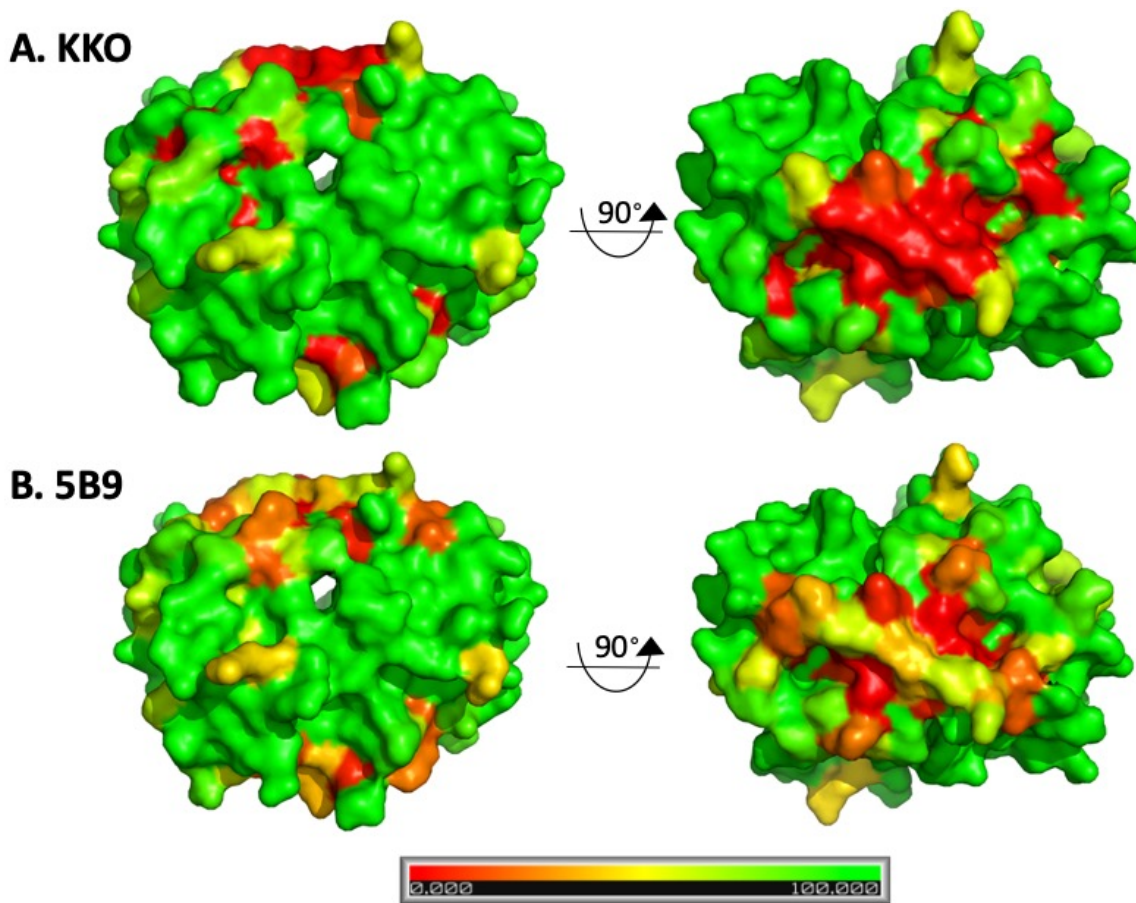
Figure 4.1: Binding of single point mutations of PF4 to KKO and 5B9. Surface representation of PF4 with binding data of PF4 mutants relative to wild-type PF4 of A) KKO and B) 5B9 imposed on 3D structure using red-green gradient. Red indicates amino acid substitution that resulted in a loss of binding and green indicates no change in binding. Modified from Protein Data Bank entry 1RHP.

BLI of KKO, 5B9, and patient sera

To determine the optimal concentrations of biotinylated-PF4 and UFH to be used

in complex, a checkerboard using different concentration combinations were tested using

an EIA binding 2 µg/mL KKO. An optimal concentration of 7.5 µg/mL of biotinylated-

PF4 complexed with 0.125 U/mL of UFH produced the highest OD_{405nm} (data not shown).

This finding suggested an optimal concentration of the PF4/heparin complex in a 60:1

concentration ratio. As a "proof of concept" KKO was utilized to measure binding to biotinylated-PF4 and biotinylated-PF4/heparin using streptavidin sensors in BLI. In addition, a non-specific monoclonal antibody Raj-1, that binds platelet glycoprotein (GP)IIbIIIa was used as a control to account for possible non-specific binding as well as a 1/10 antigen dilution of PF4 and PF4/heparin was also tested to ensure the EIA-determined experimental concentrations were detectable in this system.

Results suggest the greatest binding signal was observed for KKO binding the full concentration of either antigen. The non-specific monoclonal antibody Raj-1 produced a negligible response (Figure 4.2). The K_D values calculated using the k_{on} and k_{off} rates measured in the BLI are as predicted for murine monoclonal antibodies and for KKO are very similar between biotinylated PF4 ($K_D = 1.7 \times 10^{-6}$) and biotinylated-PF4/heparin ($K_D = 1.9 \times 10^{-6}$)(Landry et al., 2015). The values differ by one order of magnitude between full concentration and 1/10 antigen dilutions, which is expected as the k_{on} is dependent on the concentration of each binding reagent and a lower concentration would produce a smaller K_D (Figure 4.2) (Bisswanger, 2017). With the optimized assay parameters, we also tested the murine monoclonal antibody 5B9. The K_D values calculated using the k_{on} and k_{off} rates measured in the BLI for 5B9 were 9.1×10^{-10} for biotinylated PF4 and 9.5×10^{-10} biotinylated-PF4/heparin. Both K_D values for 5B9 is 1000-fold higher than the K_D for KKO.

Antigen	Analyte	Mean Response (nm)	K_D (M)
PF4	2 µg/mL KKO	1.7	1.7×10^{-6}
1/10 PF4	2 µg/mL KKO	0.7	6.1×10^{-7}
PF4	2 µg/mL Raj-1	-0.2	-
PF4/heparin	2 µg/mL KKO	1.4	1.9×10^{-6}
1/10 PF4/heparin	2 µg/mL KKO	0.5	8.5×10^{-7}
PF4/heparin	2 µg/mL Raj-1	-0.2	-
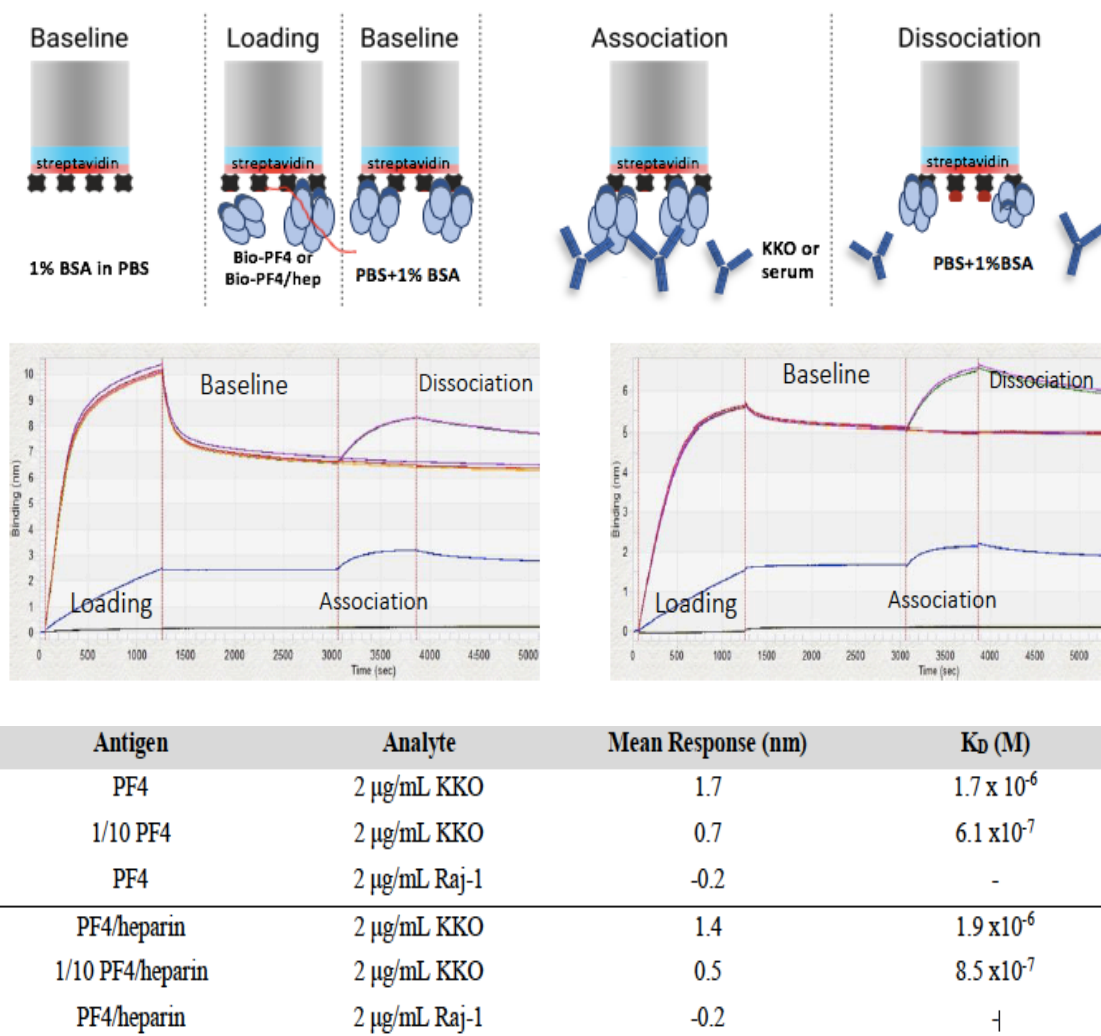
Figure 4.2: BLI spectrographs produced for KKO binding to (A) biotinylated-PF4 and (B) biotinylated-PF4/heparin. Red line represents a non-specific monoclonal antibody (Raj-1) that binds platelet glycoprotein (GP) IIbIIIa was included as a control and the blue line represents 1/10 dilution of each respective antigen. The accompanying table shows the mean binding responses and dissociation constants for KKO and Raj-1 mAbs binding to biotinylated-PF4, biotinylated-PF4/heparin and 1/10 antigen dilutions.

Current BLI literature suggests that the binding signal produced is a function of

antigen packing density and antigen size (Kumaraswamy and Tobias, 2015). To confirm

this during optimization phase, two heparin derivatives of different sizes were tested in

BLI with KKO analyte. Fondaparinux is a pentasaccharide (MW ~1.7 kDa) and

enoxaparin sodium (MW ~4.5 kDa) is a low-molecular weight heparin both of which are

smaller derivatives of UFH (MW ~15 kDa)(Rauova et al., 2006). These antigens were

subject to the same 1-hour incubation with biotinylated PF4 prior to BLI as UFH in other

experiments, and all other variables were kept constant. Results indicate loading of

biotinylated PF4/fondaparinux ($K_D = 2.05$ x 10^{-6}) produced a greater binding signal than

biotinylated PF4/enoxaparin ($K_D = 2.7$ x 10^{-6}), which was expected due to the lower

molecular weight of the former antigen (Table 4.2).

Table 4.2: Mean binding responses and dissociation constants for KKO mAb binding to biotinylated-PF4 in complex with heparin and heparin derivatives.

Antigen	Mean Response (nm)	K_D (M)
PF4/fondaparinux	1.77	2.05 x 10^{-6}
PF4/ enoxaparin	1.18	2.70 x 10^{-6}
PF4/unfractionated heparin	1.40	1.90 x 10^{-6}

Transitioning into human serum experiments, further optimization was needed given

that serum is heterogeneous and HIT antibodies are polyclonal, thus creating a potential issue

with the assay. For the first set of experiments with human serum, we tested strong SRA

positive HIT sample used in the lab as a reference in the context of HIT. Three dilutions were

tested in order to determine the most appropriate dilution for BLI and for both PF4 and

PF4/heparin antigens, antibody binding was consistent with the respective dilution strength

(data not shown). Furthermore, the serum matrix did not interfere with acquiring signal.

Once the BLI was optimized, heat inactivated serum from healthy controls (n=10), post-

surgery CPB patients (n=10) and HIT patients (n=10) were tested using BLI in duplicate. The

collective binding response data for all samples tested shows separation in binding response between the three groups (Figure 4.3). For serum samples binding PF4 the mean response (nm shift ± standard error of the mean (SEM)) for healthy controls was -0.2146 ± 0.02758; -0.02010 ± 0.02980 for post-surgery CPB patients and 0.8592 ± 0.2802 for HIT patients. Similarly, for serum samples binding PF4/heparin complexes the mean response ± SEM for healthy controls was -0.02816 ± 0.03168; 0.1541 ± 0.05888 for post-surgery CPB patients and 0.7488 ± 0.1754 for HIT patients. Using statistical analysis (unpaired t-test, two-tailed p-values), the difference between all patient groups were found to be statistically significant and determined to be the most significant between healthy controls vs. HIT and between post CPB vs. HIT for both PF4 and PF4/heparin. (PF4 p-values: post CPB vs. Healthy controls p=0.0001, HIT vs. post CPB p =0.0059, HIT vs. Healthy controls p =0.0013; PF4/heparin p-values: post CPB vs. Healthy controls p =0.0139, HIT vs. post CPB p =0.0048, HIT vs. Healthy controls p =0.0004). Notably, the mean values for HIT antibody binding biotinylated-PF4 vs. biotinylated-PF4/heparin are nearly identical, suggesting the addition of heparin as a hapten did not enhance binding of the antibodies to the PF4 epitopes in BLI. Also, there was no correlation between EIA OD values and the BLI response with either PF4 or PF4/heparin suggesting results were not based on the titer of antibody (data not shown). Finally, binding responses of serum samples from HIT patients (n=10, EIA+/SRA+) binding to PF4 and PF4/heparin were further analyzed. Specifically, HIT samples that tested neg-pos-neg and pos-pos-neg at 0, 0.1 and 100 U/mL heparin respectively in the SRA were compared. HIT samples that tested pos-pos-

neg showed generally greater binding to PF4 and PF4/heparin although this was not

found to be statistically significant (Figure 4.4).
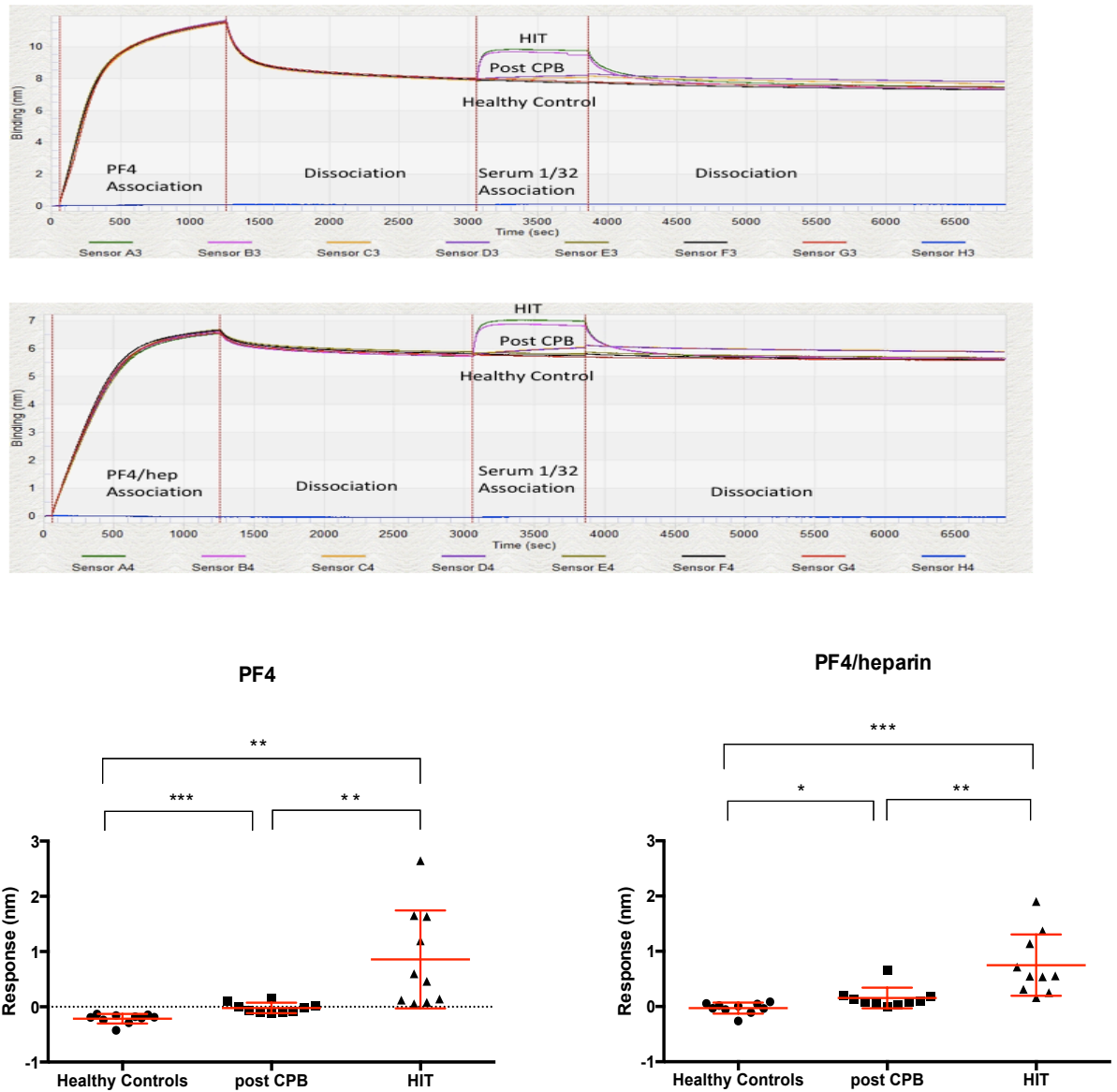
Figure 4.3: Representative and collective binding responses of serum samples from healthy controls (n=10, EIA-/SRA-), post-surgery CPB patients (n=10, EIA+/SRA-) and HIT patients (n=10, EIA+/SRA+) binding to PF4 and PF4/heparin. Overall HIT serum samples showed the highest amount of binding to PF4 and PF4/heparin compared to post CPB and healthy controls. The addition of heparin as a hapten did not enhance

binding of the antibodies to the PF4 epitopes in BLI. For serum samples binding PF4 the mean response (nm shift ± standard error of the mean (SEM)) for healthy controls was -0.2146 ± 0.02758; -0.02010 ± 0.02980 for post-surgery CPB patients and 0.8592 ± 0.2802 for HIT patients. Similarly, for serum samples binding PF4/heparin complexes the mean response ± SEM for healthy controls was -0.02816 ± 0.03168; 0.1541 ± 0.05888 for post-surgery CPB patients and 0.7488 ± 0.1754 for HIT patients. Using statistical analysis (unpaired t-test, two-tailed p-values), the difference between all patient groups were found to be statistically significant (p-value of < 0.05).
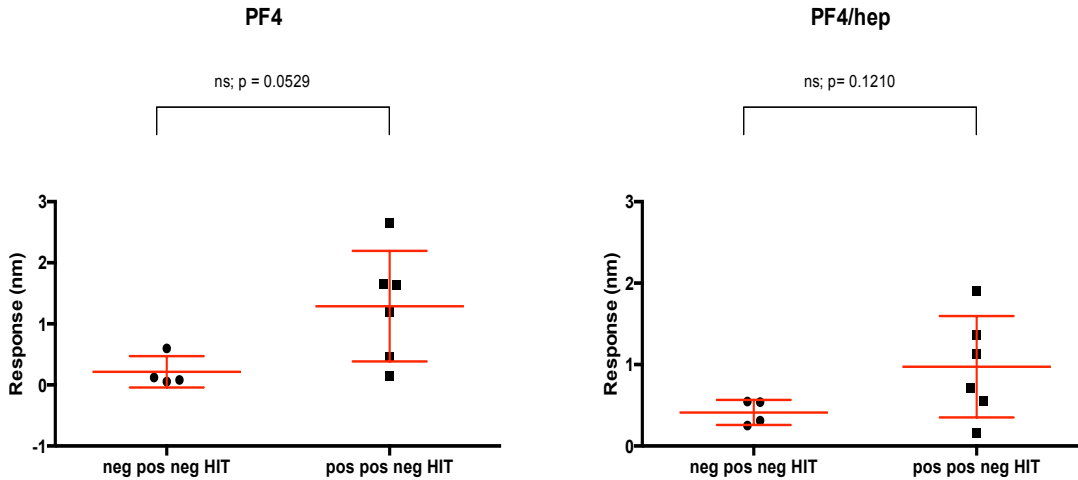
Figure 4.4: Binding responses of serum samples from HIT patients (n=10, EIA+/SRA+) binding to PF4 and PF4/heparin. HIT samples that tested neg-pos-neg and pos-pos-neg at 0, 0.1 and 100 U/mL heparin respectively in the SRA were compared. HIT samples that tested pos-pos-neg showed generally greater binding to PF4 and PF4/heparin although this was not found to be statistically significant (p-value of < 0.05).

4.6 DISCUSSION

HIT is the most common drug-induced, immune thrombotic disorder. HIT is cause by IgG antibodies that bind to a complex formed between PF4, a host protein, and heparin or cellular GAGs. Many patients exposed to heparin develop anti-PF4/heparin antibodies, yet few develop HIT. We show that in order for an anti-PF4/heparin antibody to cause platelet activation, it must bind to PF4 in a specific patch and have a high affinity to PF4.

KKO bound to both biotinylated-PF4 and biotinylated-PF4/heparin with a similar binding signal trajectory but much lower response magnitude, suggesting that because less ligand bound, consequentially there were less sites for analyte antibodies to bind (Figure 4.2). Our results support previous work showing pathogenic HIT antibodies have a similar epitope on PF4 to that of KKO and 5B9 (Cai et al., 2015, Kizlik-Masson et al., 2017). Using alanine scanning mutagenesis, we found that KKO and 5B9 bind to a specific region on PF4 (Figure 4.1). Interestingly, the alanine scanning mutagenesis showed higher degrees of binding reduction to amino acids with KKO than with 5B9. 5B9 is able to activate platelets in the SRA using lower concentrations than KKO. This could suggest that there is a threshold affinity that anti-PF4/heparin antibodies require to cause platelet activation when bound to the same patch.

Many patient sera containing pathogenic HIT antibodies display a similar binding patch on PF4 to the epitope of KKO and 5B9. Although, there is a significant reduction in binding by the antibodies with the cysteine mutants of PF4, these amino acids are not likely one of the surface amino acids that makes contacts with the anti-PF4/heparin antibodies. The reduction in binding could be due to the breaking of disulfide bonds in the PF4 tetramer when mutated. However, studies have shown that when cysteines are a part of the antigen in an antigen-antibody complex, the disulfide bonds are usually found within the center of the epitope but not making contacts with the antibody. This could be the same trend in PF4 and the region for pathogenic HIT antibodies. A number of HIT and CPB patient sera were not able to be tested in the binding assays due to their

background reactivity to bacterial lysate. This reduced our testing population and may skew the results based on patient samples with higher OD values in the EIA.

In order to ensure the differences observed were not simply due to antibody titer, HIT and CPB samples with similar OD_{405nm} EIA values were tested against one another, and statistically significant binding differences were still observed. Additionally, because serum contains a multitude of proteins (some of which are not susceptible to heat inactivation), if any non-specific binding to PF4 was occurring, a significant signal increase would be expected for the healthy controls and CPB patient groups, which was not observed (both groups produced negligible binding responses in all trials).

The ability to rationally identify the pathogenic HIT antibody site on PF4 requires deeper understanding of antibody immunodominance. Immunodominance is the complex phenomenon of unequal immunogenicity between different immunogens and different epitopes on the same immunogen (Luderitz et al., 1966). Immunodominance describes the strong tendency of the immune response to respond to complex antigens in a hierarchical manner, with higher ranking, immunodominant antigens potentially suppressing (or dominating) responses to subdominant antigens (Angeletti et al., 2017, Burton et al., 2012). In cases of HIT, anti-PF4 and anti-PF4/heparin antibodies are found within patient serum. It may be that only pathogenic HIT antibodies have a common epitope to a specific region on PF4. These pathogenic HIT antibodies could be the immunodominant

antibodies because it elicits the strongest response – platelet activation and subsequent thrombocytopenia and/or thrombosis.

Another immunological mechanism that could be applied to HIT is epitope spreading. Epitope spreading is the development of an immune response to epitopes distinct from, and noncross-reactive with, the disease-causing epitope. The ability of the immune system to attack multiple targets on a pathogen has obvious advantages. Epitope spreading can be initiated as a result of tissue damage but is also an important component of protective immune responses, acting as a method to enhance the efficiency of the immune response (Lehmann et al., 1992). Epitope spreading involves the acquired recognition of new epitopes within the same self-molecule (intramolecular epitope spreading)(Topfer et al., 1995). Anti-PF4/heparin antibodies that cause HIT have been suggested to be from an ancient immune mechanism where these antibodies are used to recognize and eliminate IgG opsonized bacteria coated with PF4 (Palankar et al., 2018). HIT then is thought to be a result of these antibodies having a cross-reactivity with heparin (Krauel et al., 2011). While only pathogenic HIT antibodies are to a specific region of PF4, the origin of antibody diversity is unknown. The phenomenon of epitope spreading as a means to increase the efficiency of an immune response against pathogens could explain the possibility of epitope spreading in HIT.

Alanine scanning mutagenesis identified a possible distinct and common area where all anti-PF4/heparin antibodies were affected by mutating PF4. Furthermore, we

used BLI to explore the binding kinetics between pathogenic HIT antibodies and non-pathogenic anti-PF4/heparin antibodies in patient sera. We determined that there is a difference between binding characteristics of patients with pathogenic HIT antibodies versus patient samples without. The role of antibody affinity to a specific area of PF4 in HIT was unclear. This work has developed our new, current hypothesis for HIT that states platelet activation anti-PF4/heparin antibodies recognize a similar region on PF4 and that the antibodies with higher affinity to this area will be able to cause Fc-mediated platelet activation that leads to HIT. The risk of HIT remains a disadvantage of heparin therapy and its life-threatening nature emphasizes the need for earlier detection and intervention. The knowledge gained from this study could develop a new diagnostic tool for HIT that recognizes antibodies specific to the region that will cause pathogenicity and have a threshold for antibody affinity that will lead to HIT.

4.7 ACKNOWLEDGEMENTS

4.8 AUTHORSHIP CONTRIBUTIONS

AH and RC carried out the described studies, analyzed data, and wrote the manuscript. IN designed the research, interpreted data, and wrote the manuscript. MI performed some experiments. DMA and JGK designed the research and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Chapter 5

The role of fluid-phase immune complexes in the pathogenesis of heparin-induced thrombocytopenia

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5.1 AUTHOR'S PREFACE

This chapter focuses on understanding the ability of fluid-phase immune complexes of PF4 and HIT antibodies to induce platelet activation. Studying PF4 mutants that had reduced binding to the platelet surface showed that fluid-phase immune complexes could activate platelets. PF4 mutants were selected based on previous work identifying important amino acids of PF4 for interaction with pathogenic HIT antibodies. Labelling of the PF4 mutants and using flow cytometry, I was able to measure the amount of PF4 mutants that bound to the platelet surface (Table 5.1). The binding data from the flow cytometry experiments were correlated to the PF4-SRA results generated by J.W. Smith showing platelet activation by the PF4 mutants (Figure 5.2). With the help of T.D. Elliott, I generated each of the 18 PF4 mutants and biochemically characterized their oligomerization. Five PF4 mutants demonstrated reduced binding but retained PF4's ability to mediate platelet activation. J.W. Smith and I analyzed the data and prepared the figures. Dr. I. Nazy, Dr. D.M. Arnold, Dr. J.G. Kelton, J.W. Smith and I wrote the manuscript.

5.2 ABSTRACT

Background/Objectives: Heparin-induced thrombocytopenia (HIT) is an adverse drug reaction caused by antibodies to complexes of platelet factor 4 (PF4) and heparin. Anti-PF4/heparin IgG and PF4 immune complexes assemble on the platelet surface and cause Fc-mediated platelet activation. Whether fluid-phase immune complexes contribute to platelet activation in HIT is not known and has implications on pathogenic mechanisms similar to other diseases characterized by circulating immune complexes. To decipher the mechanisms of platelet activation by HIT antibodies, we produced a library of PF4 mutants that allowed us to investigate platelet activation independent of platelet binding. We hypothesized that the epitopes required for PF4 binding of HIT antibodies and subsequent platelet activation can be formed in fluid-phase, without binding the surface of platelets.

Methods: We mutated 18 critical amino acids in PF4 that reduced or abolished binding by the murine anti-PF4/heparin platelet-activating monoclonal antibody (KKO). Mutant and wild-type PF4 were overexpressed in *Escherichia coli* and isolated by affinity purification. Mutant or wild-type PF4 binding to platelets was measured by incubating donor platelets with biotin-conjugated mutant or wild-type PF4 and analyzed by flow cytometry. Platelet activation was measured using the PF4-dependant ^{14}C-serotonin-release assay (PF4-SRA) in the presence of wild-type or PF4 mutants after incubating with a HIT-patient plasma or a murine anti-PF4/heparin platelet-activating monoclonal antibody (KKO). We described the ability of each mutant to 1) bind platelets; and 2) mediate platelet activation.

Results: Of the 18 PF4 mutants, only 8 were able to mediate platelet activation equivalent to wild-type PF4 despite >50% inhibition of binding to the platelet surface. The rest of the PF4

mutants: 1) bound to platelets and mediated platelet activation, similar to wild-type PF4 (n=3); 2) bound to platelets but did not mediate platelet activation (n=2); or 3) bound to the platelet surface similar to wild-type but had differential ability to mediate platelet activation (n=5) with KKO or the HIT-patient plasma.

Conclusions: Using point mutations of PF4, we identified that HIT immune complexes can be formed in fluid-phase and induce platelet activation, despite reduced binding the platelet surface. Further studies are required to investigate the role of fluid-phase HIT immune complexes in the development of thrombocytopenia, inflammation and thrombosis associated with HIT.

5.3 INTRODUCTION

Heparin is an effective, widely used anticoagulant for medical and surgical indications. Up to 3% of patients who are exposed to heparin will develop a severe drug reaction known as heparin-induced thrombocytopenia (HIT).(Martel et al., 2005, Kelton and Warkentin, 2008) HIT is caused by antibodies that form against heparin when it is complexed to the ubiquitous platelet-derived protein, platelet factor 4 (PF4, CXCL4). Once these immune complexes assemble, they can then activate nearby platelets and monocytes via the FcR, leading to a self-perpetuating cascade that results in thrombocytopenia and an intensely prothrombotic state.(Amiral, 1992, Visentin et al., 1994, Greinacher, 1994, Kelton et al., 1994, Witt and Lander, 1994, Mikhailov et al., 1999) The PF4/heparin-antibody complex activates through FcγRIIa on the platelet surface and FcγRI on monocytes, cross-linking the receptors and causing cellular activation and thrombin generation.(Chong et al., 1989, Kelton et al., 1988, Pouplard et al., 2001, Arepally and Mayer, 2001, Reilly, 2001) The increased platelet activation in HIT leads to thrombocytopenia and an increased risk of thrombotic complications that can be life-threatening (Warkentin and Kelton, 1996).

Immune complexes involve non-covalent interactions between an antigen and antibody molecules (van Oss et al., 1986, Soltis and Hasz, 1982). These fluid-phase immune complexes can be pathogenic unless they are removed by phagocytosis (Mannik, 1982). Elevated serum levels of circulating or fluid-phase immune complexes have been implicated in a number of diseases (Levinsky et al., 1977, Nydegger and Davis, 2008)

including rheumatoid arthritis and systemic lupus erythematosus, where the level of fluid-phase immune complexes correlates with disease activity (Swaak et al., 1985). The immune complexes in HIT are composed of PF4, heparin, and anti-PF4/heparin antibodies.

The epitope of anti-PF4/heparin antibodies is conformation sensitive, and additional polyanions on the platelet surface might influence the conformation of PF4 and PF4/heparin complexes or their three-dimensional structure (Padmanabhan et al., 2015a). Surface-bound PF4 is antigenic for HIT antibodies and the model HIT monoclonal antibody KKO over a narrow range of PF4 concentrations, leading to platelet activation through FcγRIIA (Rauova et al., 2006). It has been suggested that PF4 forms antigenic complexes with endogenous glycosaminoglycans (GAGs) on the surface of platelets similar to ultra large complexes that form between UFH and PF4 in solution (Rauova et al., 2005). Chondroitin sulfate has been identified as the most abundant glycosaminoglycan (GAG) found on platelet surfaces, followed by heparan sulfate (Nader, 1991). However, it has also been shown that complexes of PF4 with the GAGs on the platelet surface either exposes a different epitope or allows for better access of pathogenic HIT antibodies to its epitope (Nguyen and Greinacher, 2017). Currently, it is unresolved whether the fluid-phase immune complexes can allow the same conformational change or a different presentation of PF4/HIT IgG complexes.

Studies have yet to determine the role of fluid-phase immune complexes in HIT patients. Identifying whether these fluid-phase immune complexes are capable of initiating the Fc-mediated platelet activation found in HIT will improve our understanding of the disease. In this study, we measured the ability of wild-type PF4 and PF4 mutants to bind platelet surfaces and the immune complexes created to subsequently mediate platelet activation by anti-PF4/heparin antibodies to identify the function of fluid-phase HIT immune complexes in the pathogenesis of HIT.

5.4 MATERIALS AND METHODS

Construction, expression and purification of recombinant PF4 mutants

The full-length DNA coding sequence of human PF4 (Deuel, 1977) was cloned into the pET22b expression vector using restriction sites *Nde*I and *Hind*III (GenScript). PF4 mutants were designed where non-alanine amino acids in wild-type PF4 were mutated to alanine and the alanine amino acids in wild-type PF4 were mutated to valine. PF4 mutants were introduced into *E. coli* ArcticExpress (DE3) cells (Agilent Technologies) containing the pRARE plasmid (Novagen, Inc.). Eighteen PF4 mutants (L8A, Q9A, C10A, C12A, T16A, R22A, L27A, A32V, C36A, P37A, Q40A, I42A, N47A, C52A, D54A, L55A, L59A, and L68A) were selected for large-scale purification due to their likelihood in separating pathogenic HIT antibodies from non-pathogenic anti-PF4/heparin antibodies as determined by initial alanine scanning mutagenesis screen done previously. Overexpression of PF4 mutants was achieved by growing cultures at 37°C to

mid-exponential phase, before adding 0.5 mmol/L isopropyl β-D-1-thiogalactopyranoside (IPTG) and growing at 37°C for 3 hours.

The cells with overexpressed wild-type PF4 or PF4 mutants were resuspended in lysis buffer (20 mmol/L Sodium Phosphate, pH 7.2, 400 mmol/L NaCl, 1.4 mmol/L ß-mercaptoethanol, 5% (v/v) glycerol, 1% (v/v) TritonX-100 (Thermo Fisher Scientific), and 0.5% (v/v) DOC (Sigma-Aldrich) with protease inhibitors (Roche, cOmplete EDTA free protease inhibitor tablets), lysed by sonication, and incubated with an additional 2 mmol/L $MgCl_2$ and 10 µg/mL DNaseI for 30 min on ice. The supernatant was then cleared by centrifugation at 40,000 x g for 40 mins and applied onto a HiTrap Q HP column (GE Healthcare) equilibrated with 20 mmol/L sodium phosphate, pH 7.2, 400 mmol/L NaCl, 1.4 mM ß-mercaptoethanol, and 5% (v/v) glycerol. The flow-through of the Q HP column was then incubated at 4°C, overnight. Following the incubation, the sample was diluted 2-fold to yield a NaCl concentration of 200 mmol/L with 20 mmol/L sodium phosphate, pH 7.2, 1.4 mmol/L ß-mercaptoethanol, and 5% (v/v) glycerol, syringe-filtered with a 0.2 µM filter (Acrodisc, Pall) and loaded onto a HiTrap Heparin HP column (GE Healthcare). Contaminants were eliminated with a step gradient of 0.5 M NaCl and PF4 was eluted with a linear gradient from 0.5 to 2 mol/L NaCl. Fractions containing pure PF4 were pooled, concentrated, and buffer exchanged to phosphate buffered saline (PBS) and 1.5 mol/L NaCl. The concentration of PF4 was determined by bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). Protein expression and purity was assessed for each PF4 mutant using 4-18% SDS polyacrylamide gels.

Oligomerization of each PF4 mutant was assessed using size exclusion chromatography. After purification, 100 µg of each PF4 mutant was analyzed on a Superdex 75 10/300 GL column (GE Healthcare) in PBS and 1.5 mol/L NaCl buffer to assess multimerization.

Assessing PF4 and PF4 mutant binding to platelet surfaces

Wild-type or mutant PF4 (2 mg) was incubated with 1.25 mL of Heparin Sepharose 6 Fast Flow (GE Healthcare) packed bead volume for 1 hour with shaking at ambient temperature. EZ-Link Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific) was added to the wild-type or mutant PF4 and heparin bead mixture in 20 molar excess and allowed to react for 1 hour with shaking at ambient temperature. The biotinylated PF4 mutants were eluted from the heparin beads using PBS with 2 mol/L NaCl. Absorbance at 280 nm of each of the biotinylated PF4 mutants was measured using a spectrophotometer (BioPhotometer 6131 Spectrophotometer, Eppendorf) and used to calculate the concentration.

Biotinylation of each PF4 mutant was determined using an EIA where 10 µg/mL of streptavidin in bicarbonate buffer (pH 9.6) was coated onto microtiter plates and incubated overnight at 4˚C. The wells were washed with PBS+0.05% Tween 20 and then PBS and then blocked with 3% BSA in PBS for 2 hours at ambient temperature. 100 µL/well of 60 µg/mL biotinylated PF4 mutants diluted in 1% BSA was then added to the wells and incubated for 1 hour. Polyclonal rabbit anti-PF4 (0.1 µg/mL, LifeSpan Biosciences Inc.) diluted in 1% BSA was added to the wells and incubated for 1 hour.

After washing, alkaline phosphatase-conjugated affiniPure goat anti-rabbit IgG, Fcγ fragment specific (100 µL/well 1:5000, Jackson Immuno Research Laboratories, Inc.) diluted in 1% BSA was added to the wells and incubated for 1 hour. Wells were washed and followed by the addition of PNPP substrate (1 mg/mL) dissolved in 1 mol/L diethanolamine buffer (pH 9.6). Following a 30-minute incubation, the optical density was measured at 405nm (OD_{405nm}, Sunrise plate reader, TECAN) to assess biotinylation levels of wild-type PF4 and PF4 mutants.

To assess for the ability of wild type and mutant PF4 to bind to platelets, we prepared plates similar to that of the preparation for the standard SRA. 75 µL of platelets at 12,000,000 platelets/mL were incubated with wild-type PF4 or PF4 mutants at concentrations 0, 12.5, 25, 50, 100, or 200 µg/mL for 30 minutes with shaking at ambient temperature. Platelets were then washed once with PBS/ACD. Streptavidin-FITC (0.1 mg/mL) was added to the platelets and stained for 30 minutes at ambient temperature in the dark. Following incubation, samples were diluted two-fold with PBS before flow cytometric analysis using the CytoFLEX flow cytometer (Beckman Coulter). Human platelets were identified and gated according to the FSC and SSC. Mean fluorescence intensity (MFI) of FITC showing PF4 bound to the platelet surface for each sample was recorded and determined using FlowJo. Data were normalized, in some cases, with respect to a control experiment performed without PF4. Wild-type PF4 controls were tested to compare between independent assays.

Assessing platelet activation in the presence of PF4 and PF4 mutants using PF4-serotonin release assay (PF4-SRA)

To measure the potential for wild-type and mutant PF4 to activate platelets, we used them in a modified SRA. Platelets were labelled with ^{14}C-serotonin and prepared as described. Two PF4-SRAs were performed on different days with two different platelet donors. Labelled platelets were incubated with KKO or heat-inactivated plasma from an EIA-positive/SRA-positive confirmed HIT-patient in the presence of exogenous PF4 or PF4 mutant (0, 50, and 100 μg/mL)(Kelton et al., 1988, Sheridan et al., 1986). After a 1 hour incubation with shaking, PBS-EDTA was added and the plates were centrifuged. An aliquot of supernatant was removed from each well and added to scintillant and ^{14}C-serotonin release was measured using a scintillation counter (Topcount, Packard). KKO (10 μg/mL) and heat-activated plasma from an EIA-positive/SRA-positive confirmed HIT patient (1/64 dilution) were tested with all PF4 mutants. A ^{14}C-serotonin release $\geq 20\%$ was considered as positive platelet activation.

5.5 RESULTS

Binding of PF4 mutants to platelet surface

The majority of the PF4 mutants retained their native tetrameric structure similar to that of wild-type PF4. PF4 mutants C12A and I42A predominantly had a dimeric oligomerization in solution (Table 5.1). PF4 mutants C10A and C52A in solution are a mixture of dimers and tetramers. All other PF4 mutants purified for these experiments retained the tetramer form.

Eighteen PF4 mutants were produced in *E. coli* and their binding to the platelet surface was quantified by flow cytometry and compared to wild-type PF4. Two PF4 mutants (L8A and D54A) bound to the platelet surface at levels similar to wild-type PF4 at the same concentrations. Four PF4 mutants (L55A, L68A, C36A, and L59A) showed reduced binding (a loss of 30-50% when compared to wild-type PF4) to the platelet surface at the same concentrations. The remaining 12 PF4 mutants showed greater reduced binding ability (a loss >50%) to the platelet surface when compared to wild-type PF4 at the same concentrations, 50 and 100 µg/mL (Table 5.1).

Table 5.1: Summary of PF4 mutants and their capacity to bind to platelet surfaces

PF4 mutant	Binding at 50 µg/mL (fold MFI Increase)	Inhibition of binding compared to wild-type PF4 (%)	Binding at 100 µg/mL (fold MFI Increase)	Inhibition of binding compared to wild-type PF4 (%)
Wild-type	3.85	-	6.68	-
L8A	4.60	0	6.09	9
Q9A	2.54	51	3.04	54
C10A	1.72	55	1.98	70
C12A	1.75	55	4.54	32
T16A	1.65	57	2.22	68
R22A	1.39	64	2.30	66
L27A	1.78	54	2.98	55
A32V	1.86	61	2.27	66
C36A	2.28	54	3.63	46
P37A	2.55	33	3.11	53
Q40A	1.60	58	2.08	69

I42A	2.01	47	2.57	61
N47A	2.61	32	3.15	53
C52A	1.84	52	2.64	60
D54A	2.92	23	4.73	29
L55A	2.49	35	3.40	49
L59A	2.66	31	4.38	34
L68A	2.21	43	4.36	35

Activation of platelets with KKO and PF4 mutants

All PF4 mutants were also tested for their ability to activate platelets with the

monoclonal antibody KKO. Serotonin release was negative in the presence of C10A,

C12A and C52A using 50 and 100 µg/mL with KKO. However, the other 15 PF4 mutants

tested: L8A, Q9A, T16A, R22A, L27A, A32V, C36A, P37A, Q40A, I42A, N47A, D54A,

L55A, L59A, and L68A were able to stimulate a positive ^{14}C-serotonin release in the

PF4-SRA at both 50 and 100 µg/mL with KKO (Figure 5.1A).

Activation of platelets with SRA-positive patient plasma and PF4 mutants

The PF4 mutants were also tested for their ability to activate platelets in the PF4-

SRA with plasma from an EIA-positive/SRA-positive confirmed HIT patient containing

platelet activating anti-PF4/heparin antibodies. PF4 mutants C10A, C12A, Q40A, L55A,

L59A, and L68A mutants did not support platelet activation by the HIT-patient plasma in

the presence of PF4. In contrast, 11 PF4 mutants: L8A, Q9A, T16A, L27A, A32V, C36A,

P37A, I42A, N47A, C52A, and D54A had positive release with HIT-patient plasma in the

PF4-SRA at both 50 and 100 µg/mL of the mutant (Figure 5.1B). PF4 mutant R22A was

weaker and did not result in platelet activation at 50 µg/mL but did have a >20% release

of ^{14}C-serotonin with 100 µg/mL (Figure 5.1B).
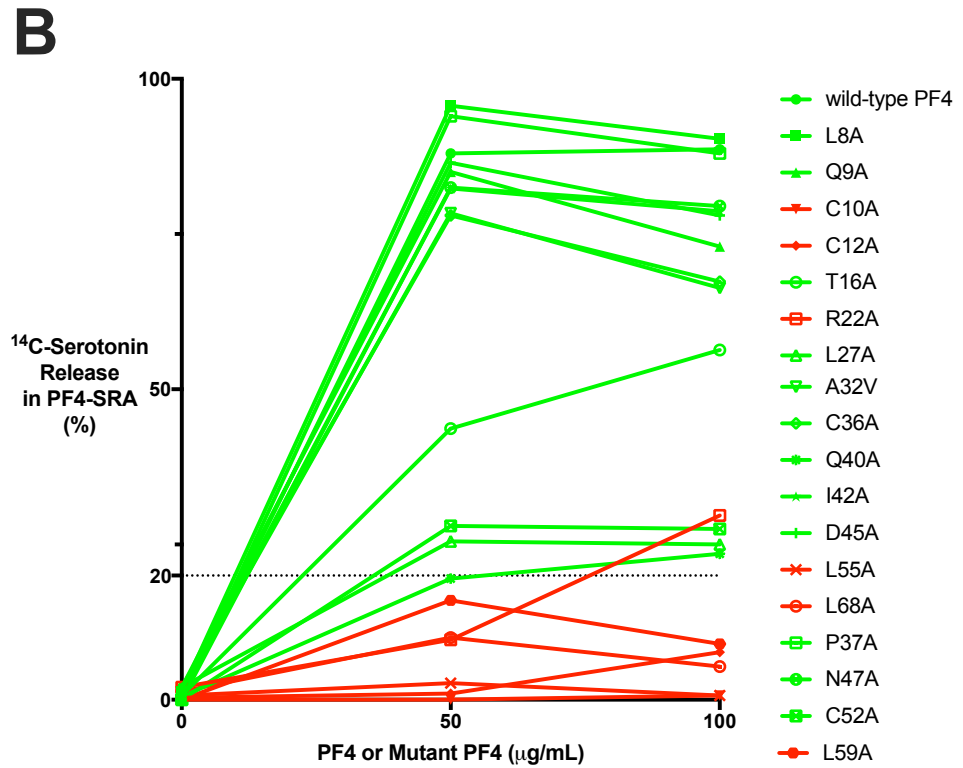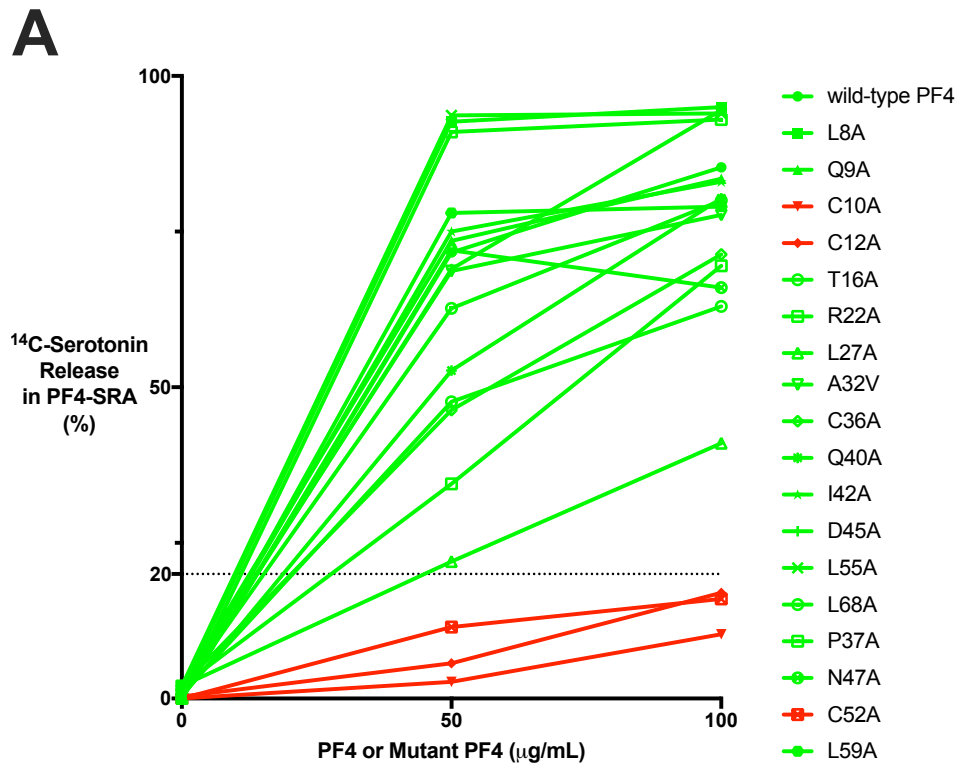
Figure 5.1: Levels of platelet activation using PF4 mutants with KKO and a HIT-patient plasma. Platelet activation by (A) KKO and (B) a HIT-patient plasma was measured in the presence of 50 and 100 µg/mL of wild-type PF4 or 18 different PF4 mutants: L8A, Q9A, C10A, C12A, T16A, R22A, L27A, A32V, C36A, P37A, Q40A, I42A, N47A, C52A, D54A, L55A, L59A, and L68A using the PF4-SRA. Of the 18 PF4 mutants tested, 15 supported platelet activation by KKO and 12 supported platelet activation by the HIT-patient plasma. Data is represented as a mean of n=2 experiments using different platelet donors.

Correlating PF4 mutants binding to platelet surface and platelet activation

The PF4 mutants that had reduced binding to the platelet surface were compared to binding with lower concentrations of wild-type PF4 than normally used in the assay. This experiment also depicted the minimum or threshold amount of wild-type PF4 binding required to induce ^{14}C-serotonin release in the PF4-SRA. We found platelet activation with KKO needed at least 12.5 µg/mL of wild-type PF4 and the HIT-patient plasma sample required as little as 6.25 µg/mL (Figure 5.2). Five PF4 mutants T16A, A32V, P37A, Q40A, I42A were able to induce platelet activation despite having reduced binding lower than the threshold binding of 12.5 µg/mL of PF4 with KKO.
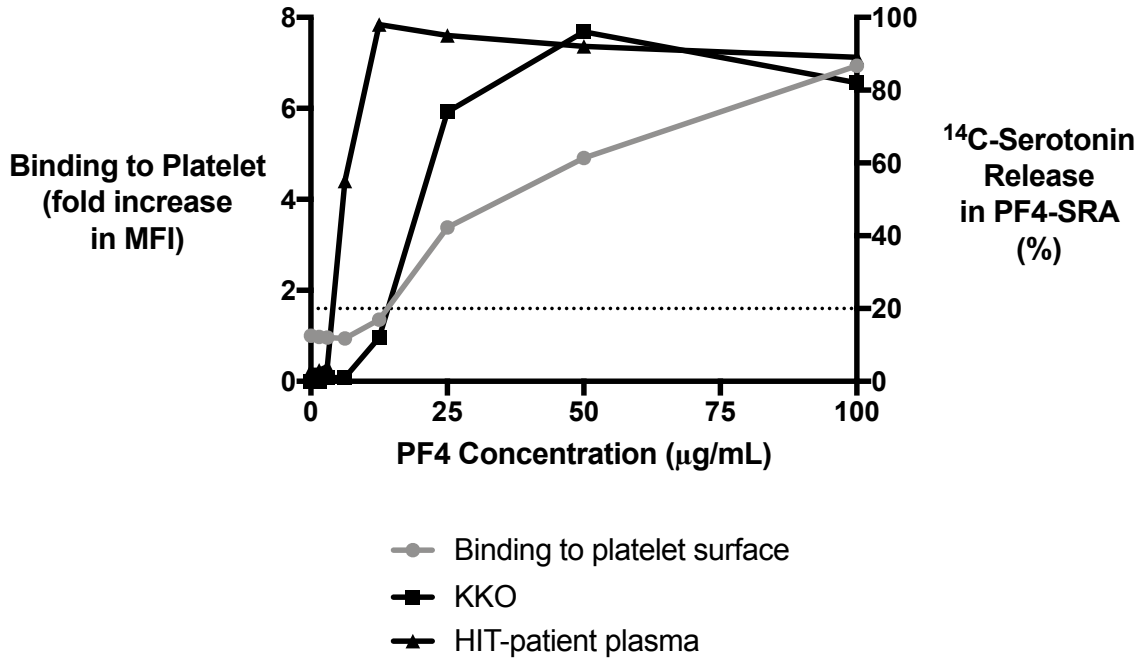
Figure 5.2: Standard curve of wild-type PF4 concentration and level of binding on platelet surface. Increasing concentrations of wild-type PF4 were tested in the PF4-SRA for platelet activation with KKO and a HIT-patient plasma. A concentration gradient of wild-type PF4 was also tested for binding to platelet surfaces through flow cytometry. The red circles and line represent the fold increase of MFI of FITC that is indicative binding to platelet surfaces and these values correspond to the left Y-axis. The black squares line represents ^{14}C-serotonin release with KKO and the black triangles line represents ^{14}C-serotonin release with HIT-patient plasma and these values correspond to the right Y-axis. ^{14}C-serotonin release >20% is considered positive in the assay for platelet activation.

Of the 18 PF4 mutants tested, 6 bound to the platelet surface and 12 supported platelet activation by KKO. Additionally, 11 of these PF4 mutants supported platelet activation by the HIT-patient plasma sample. These PF4 mutants were further characterized into three categories: PF4 mutants that bound similarly to the platelet surface as wild-type PF4 and induced platelet activation (n=3: L8A, C36A, D54A); PF4 mutants that had reduced (>50% inhibition compared to wild-type PF4) binding to the

platelet surface but induced platelet activation (n=8: Q9A, T16A, R22A, L27A, A32V, P37A, I42A, N47A); and PF4 mutants that had reduced binding to the platelet surface but did not induce platelet activation (n=2: C10A, C12A). Five PF4 mutants did not fit into the categories due to their differential reactivities with KKO and the HIT-patient plasma. Four PF4 mutants Q40A, L55A, L59A, L68A had reduced binding to the platelet surface but induced platelet activation with KKO but not the HIT-patient plasma. PF4 mutant C52A also had reduced binding to the platelet surface but only induced activation with the HIT-patient plasma, not with KKO.

5.6 DISCUSSION

HIT is a potentially fatal thrombotic disorder mediated by antibodies to complexes that form between PF4 and heparin or cellular GAGs (Kelton and Warkentin, 2008, Arepally and Ortel, 2006, Warkentin and Kelton, 1996). The role of fluid-phase in the mechanism of HIT immune complexes is currently unknown. It is currently thought that the PF4/polyanion/IgG complexes form progressively over time on the cell surface (Rauova et al., 2006). In this report, we describe the use of flow cytometry and a platelet functional assay to determine whether immune complexes of PF4/heparin can also induce platelet activation *in vitro* and as a result, induce platelet activation without being bound the platelet surface. Collectively, our study provides further insight into the immune complexes in HIT. Identifying the molecular processes of the HIT immune complex is important for a better understanding of the disease.

Our results suggest that fluid-phase HIT immune complexes can initiate platelet activation. There were five PF4 mutants that had reduced binding to the platelet surface, lower than the threshold needed with wild-type PF4 and KKO and caused a positive ^{14}C-serotonin release. Studies with the functional assays, the SRA and the HIPA, have shown that many HIT patient specimens cause platelet activation before heparin is added to the assay. This indicates the presentation of PF4 conformational neoepitopes without heparin (Prechel et al., 2005). Some studies show that surface PF4 expression correlates to the extent of pathogenic HIT antibody-mediated platelet activation (Prechel et al., 2010, Padmanabhan et al., 2015a). Our results contradict previous studies stating that optimal epitope presentation for pathogenic HIT antibodies are exhibited more prevalently on the platelet surface (Nguyen and Greinacher, 2017, Rauova et al., 2006). Binding on the platelet surface improves epitope presentation for pathogenic HIT antibodies, we show that complexes can still form in the lack of binding to platelet surface. Additionally, there have been studies that show heparin displacing the immune complexes of PF4 on platelet surface or the removal of chondroitin sulfate on platelet surfaces preventing platelet activation (Padmanabhan et al., 2015a, Cines et al., 2007). The fluid-phase immune complexes formed by the five PF4 mutants found in our experiments were able to expose the epitope needed to initiate the platelet activation found in HIT, despite reduced binding the platelet surface. With the HIT-patient plasma, platelet activation was induced at the lower levels of PF4 bound to the platelet surface.

The epitopes of anti-PF4/heparin antibodies are conformationally sensitive and additional polyanions on the platelet surface might influence the conformation of the PF4/heparin complexes (Padmanabhan et al., 2015a, Rauova et al., 2006). These results may also describe amino acids that are important in PF4 binding to the platelet surface or pathogenic HIT antibodies. Amino acids that were previously identified as integral to KKO binding were capable of causing platelet activation despite previous results abolishing KKO binding to that PF4 mutant in an EIA (Huynh et al., 2019). However, more mutations of PF4 affected the HIT-patient sample and reduced platelet activation than KKO. The high concentrations of PF4 used in this assay may overcome the loss of affinity because the loss of interaction by the single amino acid can be overcome, albeit with lower affinity.

Platelet functional assays with the addition of exogenous PF4 are becoming more prevalent in studying downstream effects of pathogenic HIT antibodies. However, there are disadvantages in using human platelets in the PF4-SRA in our study. Because we are using human platelets, when they are activated, they will begin to secrete their internal stores of wild-type PF4 that may contribute to additional activation. The concentrations of PF4 (50 µg/mL) used in our study are the optimized concentrations for KKO and HIT IgG platelet binding, and are similar to concentrations of PF4 found in the immediate environment of platelets after activation (Rauova et al., 2006). In addition, because we used patient plasma instead of purified anti-PF4/heparin antibodies from the sample, there may be some cytokines and chemokines that aid in platelet activation. Our study also

focuses solely on the platelet surface and their activation. It is possible that these soluble HIT immune complexes are not interacting with circulating monocytes and other vascular cells *in vivo*. The promotion of HIT IgG binding through the fluid-phase HIT immune aggregates can help recruit the cells also responsible for the inflammatory state and accelerate thrombin formation. It has already been shown that immune complexes of PF4 and anti-PF4/heparin HIT antibodies can activate neutrophils in the absence of exogenous heparin (Xiao et al., 2008).

Immune complexes are produced in the presence of foreign antigens. Under normal conditions, fluid-phase immune complexes are rapidly cleared by phagocytes in the liver and spleen and are of little pathologic significance. However, excessive fluid-phase immune complexes can interact with the vasculature. The excess of IgG immune complexes is a hallmark of several autoimmune diseases and is considered an important trigger of inflammation in these disorders. We propose that fluid-phase immune complexes in HIT form PF4/heparin-antibody aggregates that help initiate the platelet activation. This platelet activation allows for additional PF4 to flood into circulation and meet the levels of PF4 required for complex formation and saturation on the platelet surface. Heparin has been previously shown to promote the binding of HIT IgG to activated platelets (Newman and Chong, 2000, Horne and Hutchison, 1998). This process acts as a positive feedback mechanism, perpetuating platelet activation and additional IgG binding. However, there is no heparin used in the PF4-SRA. KKO can also aggregate PF4 and activate platelets without heparin *in vitro* (Nazi et al., 2015b, Padmanabhan et al.,

2015a). Therefore, it is possible that due to the polyclonal nature of HIT patient plasma, there exists some anti-PF4 antibodies that are heparin-independent and help cross-link PF4 molecules together to create the aggregates and fluid-phase immune complexes that can subsequently activate platelets. Once PF4/heparin antibodies have formed as a result of heparin anticoagulant therapy, the continued presence of heparin is not necessary for HIT pathogenesis. Early cessation of heparin in patients with HIT does not improve clinical outcome (Wallis et al., 1999).

This study investigated the formation of fluid-phase HIT immune complexes and their ability to induce platelet activation. Our results suggest that the soluble aggregates formed of PF4 mutants, heparin, and anti-PF4/heparin IgG act as initiators and propagators of platelet activation to produce the required plasma levels of PF4 (Mayadas et al., 2009). These increased levels of PF4 in the plasma allow for additional complex formation and potentially the initiation of downstream hallmarks of HIT: thrombocytopenia, thrombosis. In addition, our studies reveal possible amino acids not yet identified to be important for binding to the pathogenic HIT antibody. Ultimately, these findings will help lead to a better understanding of the assembly of the HIT immune complex and aid in the development of possible HIT therapeutics.

5.7 ACKNOWLEDGEMENTS

5.8 AUTHORSHIP CONTRIBUTIONS

AH carried out the described studies, analyzed data, and wrote the manuscript. IN designed the research, interpreted data, and wrote the manuscript. JWS, HB, TDE, and NI performed experiments, provided technical assistance, and wrote the manuscript. DMA and JGK designed the research and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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Chapter 6

Conclusions

This thesis focusses on a description of the antigen in heparin-induced thrombocytopenia: PF4 in complex with heparin/GAGs. Cat et al. (2015) was able to produce an X-ray crystal structure of PF4 and KKO, suggesting a probable epitope of pathogenic HIT antibodies. This work was the first to describe and account all previous studies on the amino acids possibly found in the epitope of pathogenic HIT antibodies. This knowledge gave rise to new and pressing questions: "Are the amino acids important in the epitope of KKO on PF4 similar to the pathogenic anti-PF4/heparin antibodies found in HIT patient serum?" and "What attributes of PF4 and the anti-PF4/heparin antibodies are required to produce an immune complex and cause disease?" As we started to answer these questions, additional questions arose such as "How does the antigenic complex of HIT form to cause platelet activation?" The work described in this dissertation addresses these questions and contributes to the growing understanding of the necessary elements of HIT.

The questions asked in this dissertation involved a number of experiments that required large supplies of PF4. Human PF4 is a chemokine found in α-granules of platelets. Purifying PF4 from outdated platelets received from hospital blood banks is not a sustainable source and the experimental progress would always be limited to this supply. Previous methods to express human PF4 in bacteria made numerous modifications to the native protein of PF4 to overcome the problem of low solubility of PF4. These modifications have however, led to lower yields due to refolding or creating fusion proteins that require downstream cleavage. Our expression and purification method of PF4 from E. coli (chapter 2) uses different techniques to improve the solubility of PF4 without the need for additional modifications. This body of work identified a high yield method of producing the amounts of PF4 needed for future experiments and biochemically and functionally characterizes recombinant PF4 to be similar to that of the platelet-derived PF4.

The antibody response in HIT patients is polyclonal and polyspecific, making it difficult to identify a common pathogenic epitope.(Suh et al., 1998, Ziporen et al., 1998) Previous mutagenesis studies identified amino acids D7-Q9 and amino acids in the region between the 3rd and 4th cysteines (C36 and C52) to be important in KKO binding (Cai et al., 2015, Li et al., 2002, Ziporen et al., 1998). Our previous work screening peptides of PF4 with patient sera showed that a majority of HIT antibodies recognize a noncontiguous conformational epitope on PF4 when it binds heparin (Horsewood et al.,

1996). An X-ray crystallography study has shown KKO, a monoclonal anti-PF4/heparin antibody, binds to specific amino acids on three monomers of the PF4 tetramer (Cai et al., 2015). Although studies of KKO are useful in providing models for the pathogenesis of HIT, patient sera need to be studied in order to fully define the complexity of the immune response in HIT.

With an expression and purification protocol of recombinant PF4 in place, alanine scanning mutagenesis of PF4 was then attainable. The methods outlined in chapter 2 were adapted to produce the 70 point mutations of PF4 for alanine scanning mutagenesis to study the epitopes found within HIT patient sera. Our work with alanine scanning mutagenesis of PF4 has narrowed the epitope of pathogenic HIT antibodies to specific amino acids (chapter 3). Further screening with patient serum samples, 5 amino acids were implicated as possible amino acids that could separate pathogenic HIT antibodies from non-pathogenic anti-PF4/heparin antibodies. However, because some of the amino acids suggested were cysteines, it also lends support to the detail that the tetrameric form of PF4 is integral in the formation of the HIT immune complex. In addition, our studies reveal a group of patient samples that contain subthreshold levels of pathogenic HIT antibodies that although are present, are not sufficient to lead to HIT.

Dr. Greinacher's group conducted an extensive study on anti-PF4/heparin antibodies found that certain binding characteristics determine their biological activity (Nguyen et al., 2017). Non-pathogenic anti-PF4/heparin antibodies bind with different

strengths to the HIT immune complexes than pathogenic HIT antibodies. It was suggested

that this differential binding between the groups could be either a result of differences in

their antigen recognition site or the antibodies' ability to bind with one or both Fab arms.

We determined that a binding characteristic to be considered is affinity of the anti-

PF4/heparin IgG antibody to the PF4/heparin complexes. Our work supported an idea that

potentially all pathogenic HIT antibodies bind to a specific region on the surface of PF4.

Alanine scanning mutagenesis (chapter 2) not only identified important amino acids to the

epitopes of KKO and HIT patient serum samples but when applied to the 3D structure of

PF4, create a distinct and common area where all antibodies were affected by mutating

PF4 (chapter 4). Furthermore, we used BLI to explore the binding kinetics between

pathogenic HIT antibodies and non-pathogenic anti-PF4/heparin antibodies in patient

sera. We determined that there is a difference between binding characteristics of patients

with pathogenic HIT antibodies versus patient samples without (chapter 4). The role of

antibody affinity to a specific area of PF4 in HIT was unclear. These studies developed

our new, current hypothesis that platelet activation anti-PF4/heparin antibodies recognize

a similar area on PF4 and that the antibodies with higher affinity to this area will be able

to cause Fc-mediated platelet activation that leads to HIT.

The epitope of anti-PF4/heparin antibodies is conformation sensitive, and

additional polyanions on the platelet surface may influence the conformation of PF4 and

PF4/heparin complexes (Padmanabhan et al., 2015a). It has been suggested that PF4

forms antigenic complexes with endogenous GAGs on the surface of platelets similar to

ultra large complexes that form between UFH and PF4 in solution (Rauova et al., 2005). However, it has also been shown that complexes of PF4 with the GAGs on the platelet surface either exposes a different epitope or allows for better access of pathogenic HIT antibodies to its epitope (Nguyen and Greinacher, 2017). Using platelet binding and activation studies, we found that PF4 mutants with reduced binding to the platelet surface were able to produce soluble, fluid-phase immune complexes of PF4 and anti-PF4/heparin antibodies that could induce platelet activation (chapter 5). We propose that fluid-phase immune complexes in HIT form PF4/heparin/IgG aggregates that help initiate the platelet activation. This platelet activation allows for additional PF4 to enter circulation and increase the levels of PF4 to the required for complex formation and saturation on the platelet surface.

The importance of this work extends beyond developing a comprehensive description of the necessary factors PF4, heparin, and anti-PF4/heparin antibodies must contain to lead to HIT. The information can be used to develop a more accurate and rapid diagnostic tool for HIT. Current rapid and available diagnostics tools cannot distinguish between pathogenic HIT antibodies and non-pathogenic anti-PF4/heparin antibodies. The presence of these clinically insignificant antibodies greatly increases the over-diagnosis and mistreatment of patients thought to have the disease. There is currently no rapid diagnostic test for HIT with high specificity, making the development of such an assay of crucial importance. In order to improve upon current diagnostic tools for HIT, an

understanding of how pathogenic antibodies differ from their non-pathogenic counterparts.

The long-term aim for this project will be to definitively state the necessary molecular attributes for pathogenic HIT antibodies. Mutagenesis studies and structural characterization of PF4 and model monoclonal antibodies have provided the foundation to understand the possible epitopes of pathogenic HIT antibodies. Biochemical analysis have also determined that binding characteristics of anti-PF4/heparin antibodies play a role in their pathogenicity, however, there are lingering questions. There is currently no model that fully describes the conformational change of PF4 when bound to heparin, forcing current studies to postulate surface amino acids of PF4 in interaction based on apo-PF4. To understand the amino acids that make up the epitope of anti-PF4/heparin antibodies in patient sera, the interactions of PF4 and pathogenic HIT antibodies, these immune complexes should be analyzed in the presence of heparin that is at least 11 saccharides. This aim will certainly address the current void and reveal amino acids that we overlooked and are part of the epitope of pathogenic HIT antibodies.

The characterization pathogenic HIT antibodies from this dissertation will have direct implications for patient care. This information can lead to developing an assay that accurately and rapidly identifies "true" HIT patients. In addition, it will inform key concepts in the mechanism of HIT antibodies by identifying the precise targets.

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