THE PREOPERATIVE IMMUNE PROFILE AS A PREDICTOR OF HEPARIN-INDUCED THROMBOCYTOPENIA AFTER CARDIOPULMONARY BYPASS SURGERY

CHARACTERIZATION OF THE PREOPERATIVE IMMUNE PROFILE IN A COHORT OF PATIENTS UNDERGOING CARDIOPULMONARY BYPASS SURGERY TO PREDICT POSTOPERATIVE ANTIBODY PRODUCTION AGAINST PF4/H COMPLEXES

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TITLE: Characterization of the preoperative immune profile in a cohort of patients undergoing cardiopulmonary bypass surgery to predict postoperative antibody production against PF4/H complexes

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ABSTRACT

Background: Heparin-induced thrombocytopenia (HIT) is an adverse drug reaction characterized by a lowered platelet count (50% from baseline) 4-10 days after heparin exposure. Autoantibodies specific for platelet factor 4 (PF4) bind PF4 and heparin complexes (PF4/H) and activate platelets through the FcγRIIA receptor. Severe cases of HIT can result in thrombotic complications including deep vein thrombosis, pulmonary embolism, and death.

Pathogenic class-switched antibodies against PF4/H (IgG) are detectable in circulation as early as five days post-heparin exposure and peak at 14 days. The timeline and class of antibody found in HIT patients suggest that there must be pre-existing immunity against PF4/H. Thus, B cells producing anti-PF4/H antibodies must exist prior to heparin exposure. Cardiac surgery patients are disproportionately prone to anti-PF4/H seroconversion (up to 70%) and thus are utilized in this study as a model patient group.

Research objective: The objective of this study is to determine whether the preoperative immune profile is associated with postoperative anti-PF4/H antibody production in a cohort of patients undergoing cardiac pulmonary bypass (CPB) surgery.

Materials and methods: To characterize the preoperative immune profile, we used 1) a peripheral blood mononuclear cell (PBMC) enzyme linked immunospot (ELISPOT) assay to measure the prevalence of preoperative anti-PF4/H specific antibody secreting cells (ASC) and 2) a PF4/H-dependant enzyme immunoassay

(EIA) to measure the anti-PF4/H antibodies produced by PBMCs *in vitro*. To characterize postoperative anti-PF4/H seroconversion in CPB patients, we used a PF4/H dependent EIA to measure *in vivo* levels of anti-PF4/H antibodies produced postoperatively. We also utilize a functional assay, ^{14}C-serotonin release assay (SRA) to determine if seroconverting patients produced platelet activating antibody.

Results: All patients were able to produce anti-PF4/H spots in the ELISPOT; however, this did not correlate with the titer of antibody production in vitro nor did it correlate with antibody production in the postoperative period. Instead, we found that pre-operative in vitro anti-PF4/H IgM production was associated with post-operative IgG anti-PF4/H seroconversion (Spearman's r=0.39, P=0.018). We observed that 92.1% of CPB patients produced PF4/H antibody at postoperative week 3 with some combination of IgA, IgG, and IgM. Of the anti-PF4/H seropositive patients, 26% developed platelet activating antibody and were found seropositive when the SRA was supplemented with PF4 instead of heparin, while 15.7% were seropositive in the original SRA. It was noted that 4 of 10 patients that caused the most robust platelet activation were also seropositive for anti-PF4/H IgA antibody. Lastly, throughout this serosurveillance study, several patients that demonstrated unique immunological features are presented in this study as case studies. Specifically, we report the preoperative, surgical, clinical and postoperative characteristics for 3 patients of interest: 1) in a preoperative setting, a CPB patient's PBMC were able to be activated and produce anti-PF4/H IgG antibody *in vitro*, 2)

the second patient had platelet-activating antibodies in circulation prior to intraoperative heparin challenge and early post-surgery 3) the third patient who developed probable HIT.

Conclusions: Based on our findings, we conclude that preoperative PF4/H ELISPOTs were unable to predict post-operative production of anti PF4/H antibodies. However, preoperative *in vitro* production of anti-PF4/H IgM may be associated with postoperative production of anti-PF4/IgG antibody and should be investigated further as this may help to elucidate the mechanisms for anti-PF4/H production related to HIT.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACD	Acid citrate dextrose
AF	Atrial fibrillation
AgS	Antigen stimulated
ASC	Antibody secreting cell
BCIP	5-bromo-4-chloro-3'indoylphosphate
BSA	Bovine serum albumin
CD	Cluster of differentiation
CI	Confidence interval
CPB	Cardiopulmonary bypass
DMSO	Dimethyl sulfoxide
DVT	Deep vein thrombosis
EDTA	Ethylenediaminetetraacetic acid
ELISPOT	Enzyme linked immunospot
EIA	Enzyme immunoassay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAG	Glycosaminoglycan
HiREB	Hamilton integrated research ethics board
IgA	Immunoglobulin A
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IL2	Interleukin 2
LMWH	Low-Molecular weight heparin
MVR-T	Mitral valve replacement- tissue
mAb	Monoclonal antibody
OD	Optical density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Prostaglandin E1
PE	Pulmonary embolism
PE-CY5	Phycoerythrin Phycoerythrin-cyanine 5
PO	Postoperative
PolyS	Polyclonally stimulated
PVDF	Polyvinylidene difluoride
R848	Resiquimod
RPMI 1640	Roswell Park Memorial Institute 1640 medium
SC	Subcutaneous
SD	Standard deviation
SRA	^{14}C-Serotonin release assay
SSC	Side scatter
vWF	von Willebrand factor
UFH	Unfractionated heparin

DECLARATION OF ACADEMIC ACHIEVEMENT

Research and experiments presented this study was completed by Jennifer Cui except those listed below. Julian Rubiano under the direction of Jennifer Cui, completed the supernatant EIAs. Jane Moore provided platelet-derived PF4 and assisted with some serum PF4 EIAs. Jim Smith completed all SRA tests. Tara Mcdougall consented and collected samples from CPB patients. Jennifer and Sabrina Shrestha worked in collaboration to attempt to resolve the IgM background binding in ELISPOTs.

1.0 Introduction

1.1 Platelets

Platelets, derived from the megakaryocyte lineage in the bone marrow[1], have dual hemostatic and immunological functions. Platelets are granular, anucleated cells found in circulation in a resting state, with a lifespan of 8-9 days[2]. Platelets contain three types of granules including dense (δ) granules, lysosomal (λ) granules, and alpha (α) granules, each containing distinct regulatory molecules and proteins that can degranulate in concert or at different times depending on the mechanism of activation[3]. δ-granules store small molecule mediators of vascular tone and platelet activation such as, serotonin, adenosine diphosphate (ADP), phosphate, thromboxane A_2, calcium and eicosanoids, λ granules contain proteolytic enzymes such as glycohydrolases that mediate thrombus dissolutions[3,4] and α-granules, the most abundant granule in platelets, store protein mediators of hemostatic, coagulative, reparative, inflammatory, and antimicrobial function[3,5]. Hundreds of distinct proteins have been identified in α-granules such as fibrinogen, von Willebrand factor (vWF), plasminogen, platelet-derived growth factor, and platelet factor 4 (PF4)[6]. Multiple pathways lead to platelet activation and subsequent degranulation which can result in platelet recruitment, immune cell recruitment, and initiation of coagulation cascades [7].

1.1.1 The Role of Platelets in Hemostasis and Thrombosis

In response to injury, platelets form a hemostatic plug to prevent excessive exsanguination when vascular walls are damaged, and collagen is exposed.

Platelets are tethered to exposed collagen by vWF under high shear conditions or bind collagen itself through surface glycoproteins which promotes platelet aggregation and activation[7]. Activated platelets have a direct role in the formation of thrombi[8]; however, the strength of the resulting clot is a function of platelet deposition, thrombin generation, and fibrin formation[9]. Several cell signalling and biochemical events tightly regulate the hemostatic response; delineation from these highly regulated processes through changes in platelets can cause pathological bleeding or thrombosis. Disorders causing reductions in the number of circulating platelets (thrombocytopenia) either through impaired platelet production or platelet destruction (i.e., immune thrombocytopenia, viral infections, myelodysplasia) can hinder the hemostatic process, resulting in severe hemorrhagic complications[10]. Acquired dysfunctions in platelet activation or excessive platelet deposition during thrombus formation are associated with venous occlusion[9]. Platelet dysfunction can also occur through medical interventions such as extracorporeal membrane oxygenation and cardiopulmonary bypass (CPB) [10].

1.1.2 The Role of Platelets in the Immune Response

In addition to preventing excessive loss of hemolymph, platelets have roles in inflammation and immunity. Platelets can defend against pathogens both directly, and in association with other immune cells by participating in first-line of defense to directly engulf microbes as well as releasing proinflammatory cytokines and chemotactic signals for lymphocytes. In addition, platelets can release reactive

oxygen species as well as deliver antimicrobials and kinocidin to sites of infection[11]. Platelets express host defense receptors including Fc receptors (FcγRIIA and FcεRI), complement receptor 3 (CR3) and toll-like receptor 4 (TLR4)[12] which can bind circulating immune complexes, interact with complement components (iC3b)[13], and detect LPS on the surface of gram-negative bacteria respectively. Dysfunctional platelet activity in the immune response can occur such as immune-complex induced shock, thrombocytopenia, or thrombosis[14,15].

1.2 Drug-induced thrombocytopenia

Drug-induced thrombocytopenia (DITP) occurs when drug therapies result in a decreased platelet count typically within 5-10 days of drug administration[16]. Drugs that trigger thrombocytopenia can alter the conformation of platelet surface proteins, induce production of drug-dependant autoantibodies against platelet surface proteins, resulting in antibody-mediated clearance of platelets by phagocytes through the Fc receptor[17]. Drug dependant antibody is typically transient and disappear 1-2 days after drug cessation, although if unidentified or left untreated, the risk of severe bleeding complications increases[17]. A variety of drugs can trigger DITP including chemotherapeutics, antibiotics, and sulfonamides[17]. Heparin-induced thrombocytopenia (HIT) is a well-documented DITP which can arise through exposure to the routinely used anticoagulant heparin. HIT causes thrombocytopenia, however, patients with HIT are at risk of thrombotic complications as opposed to bleeding complications.

1.3 Heparin-induced thrombocytopenia

HIT is an iatrogenic immune-mediated disease characterized by a reduction in platelet count greater than 50% from a patient's baseline value [18]. Additionally, HIT can lead to life-threatening thrombotic conditions including pulmonary embolism, and deep vein thrombosis (DVT). As a result, HIT is paradoxical in the sense that patients with HIT are at greater risk of thrombotic events despite receiving anticoagulant treatment. An estimated 5% of all patients who receive heparin during medical interventions will develop HIT, although the frequency is variable depending on the medical intervention (e.g., orthopedic surgery (5%), cardiac surgery (2-5%), ICU procedures (2%))[19,20]. Furthermore, even within a particular medical intervention, the frequency of HIT can vary. For example, hemodialysis patients are at higher risk for HIT (3.2%) within the first three months of treatment[21], while the risk lowers (0.6%) for patients undergoing chronic hemodialysis[22]. This discrepancy may indicate a reduction of risk for HIT past an acute phase of antigen exposure, inducing tolerance.

1.3.1 The Pathophysiology of HIT

HIT is believed to arise in response to the immune system recognizing antigenic complexes of heparin and PF4[23]. PF4 is a 70 amino acid (32 kDa) globular protein contained within the α-granules of platelets that are released upon platelet activation. PF4 categorizes as both a chemokine and a kinocidin for its chemotactic and antimicrobial properties[24]. In physiological conditions, PF4 exists in dynamic equilibrium with its monomeric, dimeric, and tetrameric forms. However,

PF4 predominantly exists as an asymmetric tetramer[25]. Along the surface of the tetrameric form of PF4 is a circumferential band of cationic residues, which can strongly interact with negatively charged glycosaminoglycans (GAGs), including heparin [25].

Heparin is a naturally occurring GAG commonly used as an anticoagulant. Heparin is a polysaccharide of variable length (2000-40 000 kDa) with a sulfated antithrombin binding pentasaccharide sequence at one terminus[26]. Heparin bound antithrombin leads to inhibition of thrombin that is several-fold stronger that antithrombin alone[26]. Various forms of heparin are available including unfractionated heparin (UFH), low molecular weight heparin (LMWH) and the synthetic pentasaccharide, fondaparinux. UFH is associated with higher incidence of HIT as the longer polysaccharide chain mediates formation of higher order complexes that confers greater antigenicity, though it is still widely used for its ease of reversal, cost-effectiveness, and short-half life[27].

At a given stoichiometric molar ratio of PF4 and heparin (approximately 1:1), protein surface charges are minimized so that PF4 and heparin may form PF4/heparin (PF4/H) complexes[28]. In the PF4/H complex, two PF4 tetramers are brought into proximity resulting in a charge redistribution so that the repulsive positive charge at the interface of PF4 tetramers is reduced[29]. This charge redistribution causes a conformational change in secondary structure exposing a neo-epitope on PF4[29]. The immune system recognizes this epitope in many individuals following which immunoglobulins against PF4/H complexes of class

IgM, IgA and IgG can be produced. However, the primary anti-PF4/H antibodies associated with pathogenic outcomes in HIT is IgG due to their ability to engage FcγRIIa receptors on platelets[30,31]. Once the neo-epitopes are exposed, pathogenic antibodies bind PF4/H complexes to make higher order immunogenic complexes, called ultra-large complexes (ULC). The ULC can activate platelets through immunoglobulin receptor FcγRIIA, a step that initiates a positive feedback loop of platelet activation leading to a reduction in the number of circulating platelets as well as augmenting the pro-thrombotic environment.

1.3.2 Immunological characteristics of HIT

HIT displays several unique immunological characteristics: (1) class switched anti-PF4/H antibodies (IgG and IgA), as well as IgM, can be detected in circulation within 5 days of exposure to heparin regardless of prior exposure to heparin, (2) anti-PF4/H antibodies have a short half-life, and are generally not detected in circulation 100 days post-heparin therapy, (3) after clearance of anti-PF4/H antibodies from previous treatments, re-exposure to heparin does not increase risk of HIT and (4) a previous episode of HIT does not increase risk of re-occurrence upon secondary exposure to heparin[32]. These immunological characteristics do not correspond with known classical humoral immunity mechanisms where IgM is the dominant form of antibodies produced during primary exposure to antigen, while rapid IgG production is associated with a secondary response upon antigen re-exposure [33]. Additionally, the transient nature

of HIT antibodies is unlike that of humoral immune responses which often lead to a prolonged presence of circulating IgG antibody, and in some cases can be detectable for several years post-exposure such as in vaccines like tetanus, where the half-life of circulating antibody is seven years[34]. Furthermore, there is a lack of an expected progressive immune response to subsequent heparin exposures, such as when 17 patients who had previous HIT episodes were monitored in subsequent exposure to intraoperative heparin for cardiac surgery, only one patient developed HIT[32].

1.3.3 Patient populations at risk for developing HIT

Demographics of patient populations appear to increase the risk for HIT in some cases, such as women are more at risk than men, surgical patients are more at risk than medical patients, and use of UFH confers greater risk than use of LMWH[18]. Surgical patients receiving UFH have the highest risk of developing HIT (orthopedic surgery (4.9%), cardiac surgery (1%)[19]. However, cardiac surgery patients are especially prone to developing anti-PF4/H antibodies post-surgery (up to 70%)[35]. Cardiopulmonary bypass (CPB) in surgery induces severe inflammation and can lead to substantial increases in the concentration of circulating PF4[36]. Additionally, therapeutic doses of intraoperative heparin continue to be part of best-practice guidelines for cardiac surgery as well as for post-operative reduction of deep vein thrombosis (DVT) and pulmonary embolism (PE)[37]. Unsurprisingly, given the intraoperative exposure to heparin and high concentrations of circulating

PF4, patients undergoing CPB surgery compared to other clinical populations are most likely to develop anti-PF4/H antibodies[35].

1.3.4 Identifying HIT

HIT is a clinicopathological syndrome identified in the clinic and confirmed by laboratory tests for the presence of anti-PF4/H antibodies and their ability to activate platelets. The risk for HIT is assessed clinically using a standardized set of criteria for a qualitative assessment and then translated into a score from 0-8. The test, called the 4Ts test, assigns a score based on four categories: 1) Thrombocytopenia with >50% platelet count fall from baseline, 2) timing of thrombocytopenia 4-10 days after exposure to heparin 3) development of thrombosis and 4) lack of other causes for either thrombosis or thrombocytopenia[38]. The 4Ts test scores 0-3, 4-5 and 6-8 correspond to low, medium and high risk for HIT. Due to the low predictive value of the 4Ts test, when the risk for HIT is identified, the suspicion must be ratified by positive laboratory assessments[39].

1.3.5 Laboratory assessment of HIT

HIT antibodies can be categorized as pathogenic and non-pathogenic, where pathogenicity is contingent on the ability for the antibodies to activate platelets. The pathogenic antibodies can further be divided into heparin-dependent antibodies or heparin independent antibodies (autoimmune) where the

autoimmune antibodies have a greater affinity for the PF4/H complexes. The non-pathogenic antibodies can bind PF4 and PF4/H complexes but cannot activate platelets; pathogenic antibodies can activate platelets, while pathogenic autoimmune antibodies can activate platelets in the absence of heparin. The iceberg model is used to describe the frequency of each type of antibody found in heparin exposed patients. Most people can form non-pathogenic PF4/H antibodies, fewer are able to form pathogenic antibodies, fewer still are able to form pathogenic autoimmune antibodies, and of those that can form platelet-activating antibodies, only a small percentage will develop clinical HIT and thrombotic complications. Laboratory tests that detect the presence of PF4/H antibodies and platelet activating antibodies in circulation are commonly used to confirm a diagnosis of HIT. Several methods for HIT testing have been developed including: P-selectin expression assay (PEA) (which detects p-selectin on donor platelets that were preincubated with PF4 with serum)[40]; Platelet factor 4/heparin-particle gel immunoassay (PF4/heparin-PaGIA) which separates serum samples causing agglutination by gel filtration[41]; heparin-induced platelet activation assay (HIPA) which measures platelet aggregation after platelet incubation with patient sera and heparin[42]; IgG specific chemiluminescent assay (CLIA)[43], latex immunoturbidity assay (LIA) which measures ability of patient sera to inhibit particle agglutination in the presence of PF4/H complexes[44]; PF4/H dependant EIA (PF4 EIA) detects optical density of PF4/H antibody dependant colorimetric changes; serotonin release assay (SRA) measures the release of radioactive ^{14}C-serotonin when

platelets are incubated with patient sera to quantify platelet activation[45,46]; PF4 SRA, a variation of SRA that measures release of ^{14}C- serotonin of platelets in the presence of patient serum and additional PF4[47]. The PF4/H-dependant enzyme immunoassay and other antigenic binding assays specific for PF4/H are the most commonly used to determine the presence of antibody[48]. The PF4 dependent EIA has the lowest positive predictive value for HIT, but has, however a very high negative predictive value, and is useful for ruling out a HIT diagnosis[49]. The SRA, which is widely considered to be the 'gold standard' diagnostic test, is both highly sensitive and specific, where a positive result largely confirms a suspected HIT diagnosis. However, the SRA is technically demanding and is only available to few reference laboratories due to the requirement of measuring radioactive serotonin. Therefore, other tests with shorter turn-around time and ease of processing may confer some advantages in HIT testing despite the lower positive predicted values such as the automated LIA and CLIA [43,44].

There are several challenges associated with diagnosing HIT. Firstly, utilizing sensitive but nonspecific antigenic assays lead to overdiagnosis, while underdiagnosis can result in severe thrombotic complications, including patient death. Currently, laboratory diagnosis initiates after patients are suspected of having HIT. Severe thrombotic events in real-time often occur within 30 days [50]. Rather than treatment of HIT, it would be far more effective to prevent HIT in patients at high risk for developing HIT by examining pre-operative characteristics that elevate risk for HIT.

1.3.6 Antibody production and B cells in HIT

Antibody-mediated activation of platelets is a hallmark and driving force of HIT pathology. In order to generate class switched immunoglobulins as seen in typical HIT responses (4-10 days), memory B cells that can produce anti-PF4/H antibodies are necessary. B cell immunological memory allows B cells to mount a rapid, specific response against previously encountered antigens. Specifically, long-lived plasma cells can maintain antibody levels in the serum[51], while memory B cells upon antigen re-challenge can recall antibody responses rapidly[52]. It is unclear why patients who have had no prior heparin exposure can mount immune responses involving class-switched IgG antibodies at first antigen challenge with heparin, although several hypotheses have emerged. The population of anti-PF4/H antibody-producing B cells has been hypothesized as an immunization by non-heparin polyanions. PF4 can bind various polyanions including LPS, that induce similar conformational changes to expose the neo-epitopes for antibody binding[53,54]. The previous formation of PF4 complexes with polyanions may have primed the immune system and thus established immunity against PF4/polyanion complexes preparing the immune system for production of anti-PF4/H IgG antibodies in the event of future heparin exposure. One hypothesis suggests that PF4 is a mediator between innate and adaptive immunity where PF4 may bind to a subset of bacteria so that the anti-PF4 and polyanion complex becomes targetable by antibodies[55]. Regardless of the mechanism of establishing the pre-

existing immunity against PF4/H, scientists have shown that in mouse models some B cell subsets (marginal zone B cells) are more important than others to establish immune memory[56], and that these B cells may be regulated by tolerance mechanisms which when dysfunctional can result in production of anti-PF4/H antibodies[57]. Activation of B cells to produce anti-PF4/H antibodies could also arise from complement fragments binding PF4/H complexes which have been shown to engage CD21 (or complement receptor 2) and potentially the (B cell receptor) BCR[58]. It is clear that existing immunity against anti-PF4/H complex exists in the general population; however, it is unclear if the presence of anti-PF4/H antibody producing B cells leads to production anti-PF4/H antibody after surgery or whether these antibodies, once produced, have platelet activating capacity.

2.0 Project rationale

2.1 Using the preoperative immune-profile as a predictor for HIT

The clinical features, techniques for laboratory diagnosis and immunological characteristics of HIT have been well explored, however, there is a lack of understanding of how the pre-surgical condition of a patient may contribute to post-surgical seroconversion. There is clear evidence that previous immunity exists prior to heparin exposure whether it is due to "priming" from bacterial infections such as suggested by Greinacher et al.[54], or due to dysfunction of an innate ancestral pathogen defense mechanism as suggested by Palankar et al.[55], which allows the rapid production of pathogenic IgG observed in HIT patients. Individuals who can produce anti-PF4/H antibodies do not constitutively produce them as they are generally not detectable at baseline, and individuals who have had HIT (therefore, confirmed to have a pathogenic antibody-producing B cell repertoire) do not appear to have an increased risk of HIT through subsequent encounters with heparin[32]. Thus, in order to seroconvert, B cells may be required to reach a threshold activation. This suggests, at the minimum, to acquire HIT, an individual must meet two criteria: (1) the individual has a pre-existing population of B cells with a BCR specific for PF4/H and (2) B cells must be activated to overcome any regulatory mechanisms that do not allow constitutive antibody secretion. The presence and quantity of existing B cells that produce anti-PF4/H in patients before they receive heparin therapy may affect their risk of developing PF4/H specific antibodies and therefore may be informative of an individual's susceptibility to HIT.

2.2 Research Hypothesis

In patients receiving cardiac surgery with CPB, anti-PF4/H antibody production is dependent upon the pre-surgical presence of PF4/H-specific B cells which subsequently, become activated and secrete antibodies in the surgical and postoperative environment which is required for HIT.

2.3 Research Objectives

The objectives of this study were to determine whether the abundance of anti-PF4/H antibody-producing B cells in pre-surgical peripheral blood is associated with post-surgical seroconversion to produce anti-PF4/H antibodies, and to characterize frequency and presence of platelet-activating antibodies produced by CPB patients by performing several serological tests. We used a prospective patient cohort to study the pre-operative immune profile of cardiac surgery patients receiving intraoperative heparin during the CPB procedure as heparin administration may increase their likelihood of producing anti-PF4/H antibodies. We investigated the preoperative PF4/H-specific B cell repertoire in CPB patients. To achieve this, we developed an enzyme-linked immunospot (ELISPOT) assay, to quantify the number of antigen-specific antibody-secreting cells (ASC) in CPB patients before surgery.

2.3.1 Specific objectives:

1) Develop PF4 specific B cell ELISPOT assay
2) Collect patient serum from CPB patients at pre-surgery, postoperative days (POD) 3-5, and POD 21-30.

3) Quantify the number of PF4 specific B cells before surgery
4) Characterize PF4/H antibodies in patient sera pre-surgery, and at POD 3-5 and POD 21-30 for:
    a. Seropositive for anti-PF4/H antibodies
    b. class of antibody (IgA, IgG, IgM)
    c. platelet activating antibodies
5) Determine if seroconversion post-surgery is dependent on the pre-surgical presence of PF4 specific B cells

3.0 Materials and Methods

3.1 Patient recruitment

Patients were recruited into the study who were scheduled to undergo cardiac surgery at the Hamilton General Hospital in Hamilton, Ontario. Inclusion criteria included patients ≥18 years of age with a hemoglobin of 130g/L or greater at the preoperative time who were scheduled to undergo major cardiac surgery on cardiopulmonary bypass (CPB). Peripheral blood samples were collected before surgery (day 0), 3–5 days after surgery prior to patient discharge (PO Days 3-5) and 2-3 weeks after surgery (PO week 3). Blood collected (60mL) before surgery (day 0) was drawn into sterile vacutainers of ACD and processed within 8 hours of collection. Serum and plasma samples were drawn at day 0 (10mL), between days 3-5 after surgery (10mL) and 2-3 weeks after surgery (10mL) separated, and frozen at -80°C until use. Patients were recruited in collaboration with the cardiac investigation team at the Hamilton General Hospital. Forty-five patients were recruited into the current study; however, thirty-eight were included in final analysis. Five patients did not have a PO day 3-5 sample collected but were still included in the final analysis. Some patients (*n*=5) were excluded as they did not have a PO week 3 sample collected, and two patients (*n*=2) did not complete elected surgery after recruitment. All patient-related data is presented using a non-identifying number assigned at the time of recruitment. All protocols and patient materials were approved by the HiREB.

We evaluated the patient's clinical events by tracking daily platelet counts,

anti-coagulant drug ministrations, or thrombotic events during their inpatient stay. We also tracked any readmissions to the hospital due to thrombotic complications related to the cardiac surgery. Patients who had platelet-activating antibodies at any given point was evaluated for HIT by a hematologist. All data extraction protocols and patient materials were approved by the HiREB under study number 3060.

3.2 PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from 60 mL whole blood via density centrifugation using histopaque-1077 (15mL for 25mL of whole blood) (i.e., 400g for 30 minutes). PBMCs were washed once in PBS with heparin 0.1U/mL to prevent the aggregation of cells and once in PBS alone. Both washes were centrifuged at 370g for 15 minutes. PBMCs were then re-suspended in 1mL serum-free AIM-V cell culture media and counted using a cell counter TC20, (BioRad). Cells were counted twice, and the average was taken as final count, cell viability was quantified by using trypan blue 1:1 dilution in cell count on the TC20.

3.3 PF4-specific ELISPOT assay

PBMCs from 60 mL of whole blood collected in sterile ACD vacutainers were isolated. PBMCs were then plated at a concentration of $1x10^6$ cells/mL in AIM-V culture media with PF4 (60 µg/mL), heparin (0.5 U/mL), R848 (2.5 µg/mL) and IL-2 (10 ng/mL) in sterile twenty-four well tissue culture plates. PBMC that were stimulated using R848 and IL2 are henceforth referred to as polyclonally stimulated, while PBMC that were stimulated with R848, IL2, PF4, and heparin are

henceforth referred to as antigen-stimulated unless otherwise specified. Serum-free AIM-V cell culture media was used in this study because it has been shown to minimize variation in the number of spots and optimize signal in ELISPOT[59]. Plates were then incubated for six days for optimal spot antibody secretion[60] at 37°C and 5% CO_2. While the cells were being stimulated, ELISPOT plates were prepared. PVDF multiscreen MAIPS4510 plates were hydrated with 70% ethanol and sterile H_2O five days after the PBMC isolation. The hydrated wells were then coated with 100μL of 10μg/mL anti-human IgG, IgM, or IgA antibodies in PBS or PBS alone, overnight at 4°C. Six days after the PBMC isolation the antibodies were decanted from the wells, washed three times with 200μL PBS, and blocked with PBS supplemented with 10% FBS then incubated for 2 hours at 37°C and 5% CO_2. On the same day, stimulated PBMCs were collected and washed three times in AIM-V cell culture media 370g for 10 minutes. Following the 2-hour incubation period, the plate was decanted and washed one time with PBS. Washed PBMCs were then added to the plate at 200,000 cells/well for antigen-specific and total IgA antibody wells and at 10,000 cells/well for total IgG and IgM antibody wells. Stimulated PBMCs were then seeded in four replicates for each antibody condition. PBMCs were also added to wells coated with PBS alone. AIM-V cell culture media alone was added to antibody-coated wells in duplicates. Plates were then incubated overnight at 37°C and 5% CO_2. The following day, PBMCs were decanted from the plate, and the plate was washed with a solution of PBS supplemented with 0.5% Tween-20 twice and with PBS alone three times. Next,

human platelet-derived PF4 and biotinylated human platelet-derived PF4 were diluted to 25 µg/mL and combined at a 1:1 ratio in PBS supplemented with 2% FBS. Using a mixture of human platelet-derived PF4 and biotinylated PF4 has been shown to reduce the number of background spots in an ELISPOT assay as opposed to using only biotinylated human platelet-derived PF4[61]. Three conditions were then prepared: (1) PF4 alone, which does not contain heparin, (2) PF4/heparin, which contains 20 U/mL heparin combined with 25 µg/mL of PF4 and (3) PF4 and high heparin, which contains 5000U/mL heparin combined with 25 µg/mL PF4. Biotinylated IgG, IgM, or IgA were diluted in PBS supplemented with 2% FBS and filtered through 0.2 µm syringe filters to 0.5 µg/mL. The three PF4 solutions and three biotinylated antibody solutions were then added to the plate. Once added, the plate was incubated for 2 hours at 37°C. After the 2-hour incubation, the plate was washed with TBS. Next, streptavidin conjugated to alkaline phosphatase was added to the plate and incubated for in the dark one hour at room temperature. After the incubation period, the plate was washed with TBS and the substrate, BCIP was added to the plate. The plate was incubated in the dark for 30 minutes at room temperature and washed five times with distilled H_2O. The plate was then allowed to air dry overnight at room temperature. The image acquisition of each well was performed using the ELISPOT spot counting machine Immunospot (Cellular Technologies Limited; Cleveland, Ohio, USA) while the spots were quantified using Trainable Weka Segmentation program and Image J. The frequency of antigen-specific B cells was calculated first by subtracting the

number of spots in the negative control well with no cell added from the number of spots in the antigen-specific wells. Next, we divided the mean number of antigen-specific spots by the mean number of corresponding class-specific antibody spots to determine the frequency of total antibody secreting cells (ASC). The number of spots was presented either as a frequency of PBMC seeded per well or as a frequency of total ASC. Figure 1 shows a schematic for the PF4 ELISPOT assay.

3. 4 Flow cytometry analysis of PBMC

All samples were stained with antibody bound fluorophore and incubated for 30 minutes at 4°C before analysis by flow cytometry. Isotype-matched control antibodies were used in each experiment and cells were stained so that the concentration of the isotype control antibody was equal to the concentration of the primary test antibody. Isotype controls were used to rule out non-specific binding as well as to confirm specific binding of the primary test antibody. Additionally, all experiments included compensation controls (cells stained with no stain or with only one colour) to address the spectral overlap of one fluorophore into the fluorescence spectrum of another. Compensation was accomplished manually using FlowJo Version 10.1R5 (Tree Star, Ashland, OR, USA). Monoclonal antibodies (mAb) used in this study were: CD3 PE (Thermo Fischer Scientific, Burlington, ON, Canada) , CD4 Py5.5(Thermo Fischer Scientific, Burlington, ON, Canada), CD8 APC (Thermo Fischer Scientific, Burlington, ON, Canada) CD19 Pc7 (Thermo Fischer Scientific, Burlington, ON, Canada), CD20 AF700 (Becton-Dickinson, Franklin Lakes, NJ, USA), CD21 APC (Thermo Fisher Scientific,

Burlington, ON, Canada), CD27 PB450 (Thermo Fischer Scientific, Burlington, ON, Canada), CD 38 BV610 (Becton-Dickinson, Franklin Lakes, NJ, USA) and CD138 FITC (Thermo Fischer Scientific, Burlington, ON, Canada), along with 2% FBS in PBS to total reaction volumes of 100 μL. Cells were washed with 900 μL of 2% FBS in PBS at 1250 rpm for 4 minutes, aspirated, resuspended in 300 μL of 2% FBS in PBS, and loaded into a flat bottom 96-well plate for analysis using a Beckman Coulter CytoFLEX cytometer (Beckman Coulter Inc, Brea, CA, USA).

3.5 Cells staining and gating strategy for B cells

Cells were stained with fluorochrome-conjugated antibodies for flow cytometry analysis of B cell subsets. PBMC were stimulated as previously described for 6 days in vitro. PBMCs were washed with 2% FBS in PBS twice. Flow cytometry analysis of phenotypes was used for cells at day 0 and day 6. CD19, CD20, CD21, CD27, CD38, CD20, and CD138 were used to characterize B cell subsets, while CD3, CD4, and CD8 were used to identify T cells. B cells were enumerated by quantifying cells with B cell lineage markers in the lymphocyte gate (CD19 and CD20). The gating strategy used for phenotyping were as follows: from the forward scatter (FSC) and side scatter (SSC) plot, a gate was placed around the lymphocyte population. Single cells were identified by plotting FSC-H against FSC-A and gating around the population along the diagonal. The lymphocyte gate was divided into three sub-groups that demonstrated different SSC and labelled A, B and C. In each of these gates, a Boolean gate was applied when cells were stained with a B cell phenotyping cocktail (CD19, CD20, CD21, CD27, CD38,

CD138) so that each possible combination of these fluorophores was identified. All statistics were performed using FlowJo Version 10.1R5 (Tree Star, Ashland, OR, USA). T cells were identified by gating on each lymphocyte gate (A, B and C) for cells that were positive in both CD3 and CD4 or CD3 and CD8. A schematic of the gating strategy is shown in figure S1.

3.6 Class specific anti-PF4/H EIA

Class-specific anti-PF4/heparin antibodies were measured with a PF4/heparin-dependent EIA specific for either IgG, IgM, or IgA antibodies. Ninety-six well Maxisorp plates were coated with 60 μg/mL of PF4 and 1 U/ml of heparin and underwent a one-hour incubation at room temperature. After washing, the plates were blocked with 3% BSA for two hours at room temperature. Patient or control sera diluted at 1:50, or supernatant of cultured cells without dilution to total volume 100μL/well were added to wells and incubated for one hour. After washing, alkaline phosphatase conjugated anti-human IgG, IgM, or IgA was added to wells at a 1:4000 dilution followed by the addition of the substrate, p-NPP to detect PF4/heparin-bound anti-human IgG, IgM, or IgA antibodies. UV absorbance was then measured kinetically using a microplate photometer and the OD_{405} was used to assess the presence or absence of anti-PF4/heparin antibodies. Supernatant EIA specific for IgA and IgG required longer periods of incubation with the substrate (1 hr), while serum or supernatant IgM EIA required 20 minutes of incubation with the substrate to resolve signal between positive and negative samples. For the detection of anti-PF4/heparin IgA, IgG and IgM antibodies in serum, a positive

result was an OD_{405} ≥0.45. Standard positive and negative control samples were included in each test plate. For supernatant assays, sera with known positive results were diluted into AIM-V at 1:50 and a condition with AIM-V alone were included as positive and negative controls in duplicate, respectively. The average OD plus two standard deviations from the supernatant (PBMC 6-day culture media with no additional stimulants) for each supernatant EIA (IgG, IgA or IgM) was determined as a negative control. Supernatant with an OD greater than the determined cut-off value was considered positive.

3.7 Statistical analysis

For all variables measured, the normality of data distributions was tested using the Shapiro-Wilk test. If the distribution was classified as non-normal, non-parametric statistical analyses were performed (i.e., Wilcoxon rank-sign test for paired samples, Mann-Whitney test for unpaired samples and Spearman's ρ to assess correlation. If the distribution was considered normal, parametric statistical analyses were performed (i.e., Pearson's r to assess correlation within a sample). For statistical analyses, an alpha value of 0.05 was used to assign statistical significance. All statistical analyses were performed using Graphpad Prism 7.

4.0 Results

4.1 Developing an ELISPOT Assay to determine PF4/H specific B cells in peripheral blood.

4.1.1 Quantifying Antibody Secreting Cells using ELISPOT

The ELISPOT assay is a quantitative method of showing the discrete number of secreting cells, where one spot is representative of one cell. Thus, to demonstrate that the number of spots represents the number of ASCs in our detection system, we used the hybridoma cell line KKO which constitutively secretes monoclonal anti-PF4/H antibodies in the ELISPOT. 100 KKO cells/well in triplicate were seeded onto a multititer plate and incubated overnight at 37^{o}C. To detect for cells secreting antibodies that target epitopes on PF4/H, we used PF4/H as the detection agent, and to detect the total number of cells secreting antibody of a specific class (I.e., IgG) we used class-specific antibody. Therefore, to enumerate ASC when using KKO hybridoma cells, the spots were detected using either biotinylated PF4/H complexes or biotinylated anti-mouse IgG antibodies. The number of spots formed when using the detection systems: PF4/H complexes and anti-mouse IgG (97.7 and 90.3 respectively) and the number of cells seeded were comparable, although there was greater variance in wells detected using PF4/H complexes than when detected using anti-mouse IgG antibody. Figure 2 shows representative wells of PF4/H ELISPOTs using KKO cells.

4.1.2 Stimulation of PBMC to produce anti-PF4/H antibody-secreting cells

Considering peripheral blood B cells are primarily comprised of naïve or memory B cells that do not constitutively secrete antibodies, it was necessary to determine effective ways to induce antibody secretion from resting B cells in the peripheral blood compartment. It was previously shown that R848 and IL2 used together are effective activators for increasing antigen-specific and total ASCs in PBMCs[60]. However, we found that the use of these activators while effective for increasing number of total ASC yielded a low frequency of antigen-specific spots. Therefore, we elected to improve the ELISPOT signal by using antigen in addition to the R848 and IL2 as PBMC activators. Figure 3 shows representative wells of PBMCs from a healthy individual cultured over five days in media alone, or pre-activated with polyclonal activators (R848 and IL2), with antigen (PF4 and heparin), with polyclonal activators and heparin, with polyclonal activators and PF4 and lastly with both polyclonal activators and antigen (R848, IL2, PF4 and heparin). PBMC that were not pre-activated using polyclonal activators had few to none antigen-specific spots. PBMC that were pre-activated with polyclonal activators in the absence of PF4 were able to produce some antigen-specific spots. However, wells with cells that were pre-activated with both polyclonal activators R848 and IL2 as well as PF4 had several -fold more spots than wells with cells that did not have both types of activators present. There were more spots formed when all 4 activators were used, however, the difference between polyclonal activators with PF4 or with PF4 and heparin was unremarkable. To demonstrate that the spots formed by pre-activated cells were a result of detection of ASCs and not as a result

of background binding due to PF4 added to cultured cells as an activator, we demonstrated that the antigen-specific spots increased over time. Figure 4 shows a timeline of PBMC from a healthy donor pre-activated using polyclonal activators (R848, IL2) and antigen (PF4 and heparin) together forming antigen-specific spots over 5 days. Wells shown were coated with anti-human IgM antibody and detected using biotinylated PF4/H complexes. The number of antigen-specific spots was markedly increased by day 5.

4.1.3 Lymphocyte populations in polyclonal and antigen-stimulated PBMC

To address whether the polyclonal and antigen stimulants used were effective activators of B cells in PBMC, PBMC were analyzed by flow cytometry before they were cultured, and after 6 days culturing: with media alone, with polyclonal activators (R848 and IL2) or with polyclonal activators (R848, IL2) and antigen (PF4/H). The gating strategy to find lymphocyte populations is shown in Figure S1. The lymphocyte gate contained three distinct populations that could be separated using FSC and SSC which we have labelled as lymphocyte subgroup A, B, and C. Representative distributions from one healthy donor for each of these populations are shown in Table S2. We found that in the presence of polyclonal or antigen activators, the percentage of lymphocytes in subgroup A and C were increased when compared to PBMCs from culture day 0 or when cultured over six days with media alone. However, polyclonal activators increased the percentage of lymphocytes in subgroup C (37%) more than A (17%), while including antigen as an activator increased the percentage of lymphocytes in subgroups A (38%)

more than subgroup C (20%). PBMC were stained with a cocktail of fluorophore-conjugated phenotyping antibodies against CD19, CD20, CD21, CD27, CD38, and CD138 to differentiate B cell subsets and CD3, 4 and 8 for T cells using flow cytometry. The B cell phenotypes were gated using a Boolean gating strategy to evaluate every possible combination of differentiation marker. This gating strategy combined positive gates of the 6 B cell differentiation markers used to generate 64 combinations that cells could express these markers. All populations identified using this gating strategy that had greater than 100 events are shown in Table S2.

4.1.4 Expression of B cell lineage markers on polyclonal and antigen-stimulated PBMC

B cell lineage markers CD19 and CD20 were evaluated in each of the culture conditions as well as in each subgroup (*n*=1). Figure 5a shows the percentage of all lymphocytes that were positive for CD19 in each culture condition. It was found that using antigen as well as polyclonal activators resulted in the highest number of CD19+ cells (31%) within the lymphocyte gates when compared to polyclonal activators (21%), media (3.6%) or with fresh PBMC that were not cultured (4.6%). Figure 5b shows the percentage of cells in each lymphocyte subgroup that were CD19+. In each of the cultured cell conditions, subgroup C had the greatest proportion of CD19+ cells, while subgroup B had the smallest proportion of CD19+ cells. Figure 5c shows the percentage of all lymphocytes that were positive for CD20 in each culture condition. Pre-activated PBMC with either polyclonal activators (33%) or antigen (30%) resulted in the highest percentage of CD20+ cells compared to cultured cells without activators or fresh PBMC. Figure

5d shows the percentage of cells in each lymphocyte subgroup that were CD20+ cells. In each of the cultured cell conditions, subgroup C had the highest percentage of CD20+ cells while subgroup B had the smallest proportion of CD20+ cells. Subgroups A and C contained the majority of B cell lineage cells, while subgroup B was comprised mainly of T cells (Table S2).

4.1.5 IgM bind non-specifically to PVDF membrane

The ELISPOT detection system in this study used biotinylated antigen to identify bound antibodies to quantify antigen-specific spots. To ensure that spots formed in antigen-specific wells in the ELISPOT reflect the class of antibody that was to be captured, we checked if there was any background binding of other antibody classes when capturing one class of antibody (i.e., coating with antihuman IgG capture antibody should result in only bound IgG and no spots should be detectable if detecting with antihuman IgA antibody). Figure 6 shows representative wells from PVDF membrane multititer plates coated with monoclonal anti-human antibodies for IgA, IgG, and IgM which were detected with antibodies that were not a matched pair against the capture antibody in replicates of eight. We seeded R848 and IL2 pre-activated PBMC from healthy controls at 10^5 cells/well overnight and detected bound antibody using antihuman IgA, IgG, and IgM for each type of capture antibody used. It was found that when detecting spots using antihuman IgA and IgG antibody, spots only formed when the capture antibody and the detection antibody were a matched pair. However, when antihuman IgM antibody was used as the detection, spots were detected when the

coating antibody was against its matched pair, IgM (270 ±24.6) but were also detected when the coating was against human IgA (71.8 ±10.8) and IgG (48.4 ±10.6). Spots also formed in wells detected using anti-human IgM that did not have any capture antibody (82.8 ±11.4). Changes in buffer, detection antibody companies, blocking agent and membrane plates did not decrease the binding of IgM antibodies to the membrane and forming spots (Table S4). Therefore, we were unable to ensure that spots detected were class specific for IgA or IgG and all reported antigen-specific ELISPOT results are for antibody class IgM.

4.2 Serosurveillance of cardiac surgery patients

4.2.1 Rates of preoperative and postoperative anti-PF4/H antibody production

We analyzed 38 patients at three time points: before surgery (pre-surgery), post-operative (PO) days 3-5 days after CPB surgery and approximately PO week 3 (range 14 – 35 days). Table 2 demonstrates the baseline demographics of the cardiopulmonary bypass patient cohort included in the final analysis. CPB is known to induce the post-operative production of anti-PF4/H antibodies[35]. Figure 7 shows the number of patients who tested positive for anti-PF4/H antibodies before and after CPB. Using class-specific PF4/H dependent EIA we found that pre-surgery, 29% of patients tested positive for anti-PF4/H antibodies which can be further broken down into patients that were positive for IgA (*n*=7, mean OD=0.76 ±0.25), IgG (*n*=5, mean OD=0.67 ±0.18), and IgM (*n*=5, mean OD=0.66 ±0.11). Little change was noted at PO days 3-5 compared to pre-surgery; however, 92% of

patients developed anti-PF4/H antibody at PO week 3. Table 3 shows the number of patients that developed either IgA, IgG, IgM or combinations of these immunoglobulin classes against PF4/H. Most patients were positive for IgG antibodies (*n*=27), followed by IgA (*n*=19) and IgM(*n*=15). Most commonly, patients developed anti-PF4/H antibody of the IgG class only (*n*=11, 28.9%), or developed all three classes of antibody (*n*=10, 26.3%), a small number of patients did not develop any anti-PF4/H antibody (*n*=3, 7.9%). PO week 3 was when most patients had detectable levels of anti-PF4/H antibody, and thus was used for subsequent analysis that included post-operative antibody production.

4.2.2 Rates of postoperative platelet activating antibody production

Platelet activation by anti-PF4/H antibodies is considered a hallmark of HIT. We show here that many patients develop platelet-activating antibodies at PO week 3, but do not develop HIT. Platelet-activating antibody was detected using the functional assay measuring platelet activation, the SRA, as well as a variant of the SRA that requires the addition of exogenous PF4 as previously described by our group [47]. The PF4 SRA is a more sensitive test for antibodies that have platelet activating capacity which can detect platelet activating antibody at subthreshold levels for HIT. The majority of anti-PF4/H antibodies in CPB patients developed at the PO week 3 time; therefore, sera from this time point were investigated for platelet-activating antibody using the SRA and PF4 SRA. Figure 8a shows the individual percentage of platelet activation of 38 CPB patient sera samples taken

at PO week 3 in the PF4 SRA with different concentrations of exogenous PF4 added (0μg/mL, 50μg/mL and 100μg/mL). Figure 8b shows the corresponding data for the SRA with different concentrations of heparin added (0U/mL, 0.1U/mL and 0.3U/mL). The test was considered positive when the platelet activation was greater than 20% of control. Figure 8c shows the percentage of platelet activation in the 10 patients (26.3 %) found positive in the PF4 SRA. Figure 8d shows the percentage of platelet activation in the SRA for the 10 patients that were positive in the PF4 SRA, of which 6 (15.8%) were positive for the SRA. Of note, strongly platelet activating samples in the PF4 SRA did not necessarily result in a parallel result in the SRA.

4.2.3 Platelet activation by CPB patient sera is related to antibody class

Figure 9 shows the relationship between the percentage of platelet activation in the SRA and OD in the PF4 EIA using sera from post-operative CPB patients. Figure 9a showed that patients who had platelet activating sera (shown as open dots) were all seropositive in the IgG EIA. PF4 SRA seropositive patients (figure 9, row 1) most commonly produced only IgG antibody (*n*=5, 50%), followed by patients who produced IgG and IgA (*n*=3, 30%), IgG and IgM (*n*=1, 10%), and lastly all three classes (*n*=1,10%). The six of 10 patients that were also positive in the SRA (Figure 9a, row2) were either IgG positive (*n*=3, 50%), IgG and IgA positive (*n*=2, 30%) or positive for all three classes (*n*=1, 20%). Of the patients who had platelet-activating antibodies, the IgA seropositive patients consistently had greater platelet activation (p=0.0095) than patients who were IgA seronegative in

the PF4 SRA (figure 9b). A similar trend is seen in the SRA although this was not statistically significant (p=0.10) (figure 9c). Additionally, we examined if the pre-surgery presence of anti-PF4/H antibodies resulted in post-operative production of platelet-activating antibodies. It was found that patients that were positive in the PF4/H dependent EIA pre-surgery did not produce platelet activating antibody post-surgery (Figure S2).

4.3 Measuring the relationship between the pre-operative frequency of PF4/H-specific ASCs and post-operative anti-PF4/H antibody production

4.3.1 Characterization of pre-surgery anti-PF4/H ASCs in CPB patients

We used the PF4/H ELISPOT to quantify PF4/H specific B cells prior to surgery. Figure 10a shows when preoperative CPB patient PBMC are pre-activated, IgM ASC are the most prevalent. IgM specific ASC from PBMC activated with polyclonal activators displayed a normal distribution (Shapiro-Wilk's W=0.96, P=0.2). When comparing the effect of polyclonal pre-activation (*n*=37) to polyclonal and antigen-specific pre-activation (*n*=18) the total number of ASC are significantly increased for IgA (p=0.006), but significantly decreased for IgG (p=0.03) and IgM (p<0.0001). Figure 10b shows that the number of IgM PF4/H specific ASC are both normally distributed, but significantly different in different culture conditions, where polyclonal (W=0.96, P=0.19) and antigen-specific (W= 0.93, P=0.16) pre-activators used together resulted in significantly greater production of IgM specific PF4/H spots (p=0.017). The frequency of the IgM spots was determined by dividing the number of specific IgM spots by the number of total IgM spots after activation. Figure 10c shows that, as expected from the decrease in total IgM ASC and

increase in specific IgM PF4/H ASC, the frequency of IgM PF4/H specific spots are significantly greater when PBMC are pre-activated using polyclonal and antigen-specific activators ($p<0.0001$), the distribution of antigen-specific spots remains normal ($W=0.92$, $P=0.13$). When IgM PF4/H specific spots are compared to total IgM ASC, there is a weak positive correlation (Spearman's ρ (95%CI) =0.44(0.11,0.68), P=0.009) for polyclonal pre-activated PBMC and slightly stronger positive correlation (Pearson's r(95%CI) =0.67(0.30,0.87), P=0.002) for antigen-specific activated PBMC. This correlation suggests that of total IgM ASC, a greater proportion of IgM ASC are PF4/H specific when pre-activated with PF4/H, R848, and IL2 as opposed to R848 and IL2 alone. In addition, we assessed total class specific anti-PF4/H specific antibody production by pre-activated PBMC using the supernatant of culture media of PBMC that were incubated with polyclonal and antigen stimulants for six days. Figure 11 shows the OD of class-specific PF4 EIA from supernatants of PBMC cultured with or without pre-activation. For each antibody class, the mean and standard deviation of PBMC cultured for six days without pre-activation were used to determine a positive OD cut-off value which is summarized in Table S5. PBMC pre-activated with R848 and IL2 produced the most anti-PF4/H antibody for IgA and IgM. Polyclonally activated PBMC had a slightly greater mean OD than PBMC stimulated with antigen($p=0.03$) or cultured in media ($p=0.004$), however, due to a large overlap between OD ranges in the IgG PF4 EIA there was a poor separation between supernatant of activated cells and not-activated cells. One patient was able to produce IgG anti-PF4/H antibody when

their PBMC were pre-activated regardless of activating agents used. This patient developed weakly platelet antibody postoperatively (Figure 8) (patient no. 73). IgM anti-PF4/H antibodies were produced most frequently (55 of 57, 96%) and most abundantly (mean OD=1.56 $\pm$0.98) when PBMC were pre-activated with R848 and IL2; this was also normally distributed (W=0.97, P=0.11).

4.3.2 Preoperative PF4/H-specific ASC and class-specific post-operative anti-PF4/H antibody levels

To identify if preoperative PF4/specific ASC were related to postoperative production of anti-PF4/H antibodies, we compared the number of preoperative IgM PF4/H specific ASC, and production of anti-PF4/H antibodies by pre-activated PBMC to the postoperative production of anti-PF4/H antibodies and production of platelet-activating antibodies. Figure 12 shows that preoperative IgM PF4/H spots do not correlate to the postoperative production of anti-PF4/H antibodies of any class regardless of pre-activating agents used. We also compared the production of PF4/H antibodies by activated PBMC to postoperative production of anti-PF4/H antibodies for each of IgA, IgG, and IgM. Figure 13 shows that pre-operative production of IgA and IgG antibody by R848 and IL2 activated PBMC did not correlate to postoperative production of anti-PF4/H antibody of any class. However, when pre-operative production of anti-PF4/H specific IgM by polyclonally activated PBMC was compared to postoperative production of anti-PF4/H specific IgG, there was a positive correlation (Spearman's ρ (95%CI) =0.39(0.06,0.065), P=0.018).

Comparison of antigen-activated PBMC supernatant and post-operative production of antibodies was also considered, however, there was no significant correlation (Figure S3).

4.4 Case studies

4.4.1 Production of IgG anti-PF4/H antibody by activated PBMC from pre-operative CPB patient

When preoperative PBMC from patient 73 were cultured, IgG specific anti-PF4/H antibody was found in PBMC culture media (supernatant fraction) using the PF4/H dependent EIA (OD=0.74) when activated using R848 and IL2 with or without PF4/H, while culture media from PBMC without activators added was negative (OD= 0.05, IgG supernatant OD cut-off=0.24, table S3). The antibody found in the supernatant was not platelet activating in the PF4 SRA or SRA (Table S6a). PBMCs were isolated and analysed using the PF4/H specific ELISPOT preoperatively and at PO week 3. Production of PF4/H specific IgM spots was not significantly different before or after surgery (Table S6a). This patient was suspected for HIT at PO day 2, however, did not have detectable anti-PF4/H antibody and did not develop any thrombotic complications post-surgery. It was improbable that they had HIT based on lack of complications and negative PF4/H dependant EIA at the time of testing. Figure 14 shows patient 73's clinical course.

4.4.2 Presence of platelet-activating antibodies in CPB patient pre-surgery

Platelet-activating antibody was found in 10 of 38 CPB patients at PO week 3. To investigate the development of platelet activating antibody over time in these

patients, the pre-surgical and PO day 3-5 sera were used in the PF4 SRA and SRA. It was found that platelet-activating antibody developed only at the PO week 3 time point for 9 of 10 patients (Figure S4). Patient no. 58 however, had detectable platelet activating antibody pre-surgery (PF4 SRA [100μg PF4/ mL] platelet release =46%) and PO day 3-5 (PF4 SRA [100μg PF4/mL] platelet release = 58%). This patient went on to develop strongly platelet activating antibody at PO week 3 (PF4 SRA [100μg/mL] platelet release = 84%, SRA [0.3U/mL UFH] platelet release = 70%). This patient was considered seronegative in the IgG specific PF4/H dependant EIA pre-operatively (OD=0.42), and at PO day 3-5 (OD=0.39). IgA and IgM specific PF4/H dependent EIA were also negative at all time points. However, at PO week 3, it became strongly seropositive for IgG (OD=2.6). This patient experienced thrombocytopenia on day 2 that was greater than 50% baseline (platelets = 61 $x10^9$/L) however, quickly recovered platelet count by PO day 5 (platelets = 197$x10^9$/L). This patient was not suspected of HIT and did not develop postoperative thrombotic complications. The clinical course for this patient is depicted in figure 15, and the detailed test results for PF4 EIA, (PF4) SRA and ELISPOT are summarized in Table S6b.

4.4.3 Patient who developed probable HIT

Of 38 CPB patients, one patient (patient no. 64) developed probable HIT. This patient's platelet count decreased to 79$x10^9$ /L by PO day 3 however, recovered platelet count to 137$x10^9$ /L by PO day 5 and was discharged from the hospital. On PO day 10, this patient was readmitted with atrial fibrillation and

stroke, and was restarted on LMWH by PO day 11 when platelet count was $165x10^9$/L. There was a lack of thrombocytopenia which may have been due to a blunted platelet count recovery[62]. This patient's clinical course is depicted in figure 16, and detailed test results for PF4 EIA, (PF4) SRA and ELISPOT are summarized in Table S6c.

5.0 Discussion

HIT is an immune-mediated prothrombotic adverse drug reaction occurring in approximately 5% of patients who receive heparin therapy[63] HIT antibodies are transient and not constitutively present in circulation, however up to 70% of cardiac patients produce anti-PF4/H antibodies[63]. In this study, we investigated the complete picture of serological transformation in CPB patients by characterizing pre- and post- cardiac surgery immune events by looking at the preoperative presence of PF4/H-specific ASC, antibody production by *in vitro* stimulation, and characteristics of postoperative anti-PF4/H antibody production.

5.1 Preoperative ASC

In this study, we have established and used an ELISPOT assay to detect and quantitate PF4/H specific B cells in peripheral blood from CPB patients before cardiac surgery. Our results showed that the ELISPOT assay could detect and quantitate anti-PF4/H IgG hybridoma cells and IgM producing B cells by *in vitro* stimulation of PBMC from peripheral blood of pre-surgical CPB patients. We were able to induce PF4/H specific ASC expansion in all patients using polyclonal PBMC stimulant (mean frequency/10^5 PBMC=96.4, range [min, max] =7.7, 216). Antigen stimulation of the B cell receptor (BCR) is known to be important for the proliferation and differentiation of B cells[64] and has been previously used to expand antigen-specific B cell subsets in ELISPOT [65]. Therefore, in an effort to augment the PF4/H specific ASC population in cultured PBMC, platelet-derived PF4 and heparin were added to PBMC for additional B cell activation. When antigen was added as a

stimulant as well, a greater number of PF4/H specific spots were detected (mean frequency/10^5 PMBC [min, max] = 165 [10,347]). We found that PBMC activated simultaneously with polyclonal activators and antigen produced markedly increased number of antigen-specific spots by day 5 when compared to PBMC cultured in media alone or with polyclonal or antigen activators alone. PBMC cultured in different polyclonal activators were further characterized by flow cytometry. We found that there were three populations in activated PBMC distinguished by cell size and granularity. In particular, we confirmed that cells cultured with R848 and IL2 alone or with PF4/H had increased proportions of cells presenting B cell lineage markers CD19 and CD20 as expected based on increased number of total ASC. R848 IL2 activated PBMC preferentially increased the proportion of lymphocytes in subgroup C. While R848 IL2 and PF4/H activated PBMC showed an increased proportion of lymphocytes in both subgroup C and subgroup A which were more granular cells. The frequencies we found for PF4/H specific ASC are greater than previously reported results from Zheng et al.[57] in healthy controls. IgM ASC specific for PF4/H from healthy controls were estimated using a statistical method to calculate the most probable number (MPN) based off of serial dilutions of *in vitro* stimulated cells. Their method assumed that detectable antibody by PF4 EIA was representative of at least one anti-PF4/H secreting cell. Therefore, their reported range of 0.1-1.0 PF4/H specific B cells per 1000 B cells in healthy donors is minimized. Our frequencies of antigen-specific spots were also greater when compared to other B cell ELISPOTs studies using *in vitro* pre-

activated B cells. For example, in patients with pemphigus vulgaris (PV), in an ELISPOT for memory B cells, 0-8.4 ASC were detected per 10^5 PBMC[65]. In a study surveying postoperative cardiac surgery patients, anti-PF4/H spots were detected in 2 of 30 patients with a frequency of 3-4 spots per 10^5 cells [66]. However, in both the PV patients and post-cardiac surgery patients' cohorts, ELISPOTs were used to detect IgG specific ASC which we have shown have lower frequencies in peripheral blood compared to IgM ASC. Lastly, our method of B cell activation uniquely uses polyclonal stimulants R848 and IL2 as well as specific HIT antigen, PF4/H, rather than LPS, CpG, pokeweed mitogen which are other commonly employed cell stimulants for B cell activation. R848 and IL2 were previously shown to be a highly potent B cell activator and could markedly increase the number of spots formed when used in an ELISPOT [60].

5.2 Preoperative anti-PF4/H antibody

We demonstrated that consistent with the frequency of PF4/H specific IgM ASC detected, when we tested for anti-PF4/H antibody in supernatant fractions from culture media of cells stimulated for ELISPOTs, IgM antibody was detected in 96% of R848 and IL2 activated PBMC and 83% of R848, IL2 and PF4/H activated PBMC. However, the number of spots and the frequency of antigen-specific ASC did not relate to overall antibody production found in supernatants when tested in the PF4 EIA. On average, OD in the PF4 EIA of the supernatant was significantly greater than antigen-stimulated PBMC for all three immunoglobulin classes: IgA ($p<0.0001$), IgG($p=0.03$) and IgM ($p<0.0001$). The discrepancy between the

number of antigen-specific ASC and the amount of detectable antibody in the supernatant may be due to the number of total cells and cell viability in culture. Cells that were cultured with R848 and IL2 tended to have higher viability by culture day 6. Cells that were cultured using PF4/H alongside R848 and IL2 were less viable and had a smaller absolute number of cells at the end of culture day 6. Therefore, the discrepancy between antibody titre and ASC detected in the ELISPOT may be attributed to a lower concentration of ASC in antigen-activated cells. In our hands, although using PF4/H as a stimulant helped to activate PF4/H specific cells as measured using the ELISPOT, using the supernatant fraction of cells cultured in media with R848 and IL2 was more suitable for downstream analysis of anti-PF4/H antibody production in pre-operative CPB patients. Of the R848 and IL2 activated PBMC, 43% were positive for IgA, and 11% were positive for IgG. Determining IgG positive PF4 EIA using supernatant was especially challenging as there was very minute, though statistically significant (Mann-Whitney test, P=0.006), differences between positive and negative PF4 IgG EIA OD. Despite statistically significant differences, the overlap between what constituted as a positive and negative OD for IgG in supernatant calls for scrutiny of each positive result determined based on this cut off (cut off = 0.23). One particular patient (patient no. 73) was of interest as they were able to produce anti-PF4 IgG antibody at high titre (OD=0.74) using PBMC before heart surgery, which is the first known demonstration of immune memory for HIT-related antibodies.

5.3 Postoperative seroconversion

Changes in antibody production were monitored in the CPB patient cohort by sampling sera from pre-surgery, 3-5 days post-surgery and 3 weeks post-surgery prospectively. We studied time of seroconversion, immunoglobulin class, immunoglobulin levels and capacity to cause platelet activation. We found that a greater than previously reported proportion of cardiac patients (up to 70%), developed anti-PF4/H antibodies[63]. Only 3 of 38 (8%) patients did not produce any class of anti-PF4/H antibody post-surgery, 3 weeks after intraoperative heparin exposure. Seroconversion occurred in the majority (92%) of patients after they were discharged from the hospital. Seroconversion found in patients three weeks after surgery but not during the inpatient period is expected as previous studies have shown that anti-PF4/H antibodies of IgA, IgG, and IgM develop at a median of 6 days after first exposure to heparin [67] while cardiac patients are discharged as early as 3 days post-surgery. We found that as previously reported, anti-PF4/H IgG seroconversion was the most prevalent followed by IgA and IgM. By week 3 post-surgery, 27 of 38 (71%) patients developed anti-PF4/H IgG antibody. When we tested these sera using the PF4 SRA and SRA, we found that 10 of 38 (26%) and 6 of 38 (16%) were platelet-activating in each functional assay respectively. The SRA is known to have high specificity and sensitivity for HIT and is often used to confirm HIT diagnosis, while the PF4 SRA has been shown to be more sensitive but less specific for disease-related platelet-activating antibodies [47]. Nonetheless, the PF4 SRA is valuable in demonstrating the presence of platelet-activating anti-PF4/H antibody despite these antibodies being clinically irrelevant.

5.4 Platelet activating antibodies in CPB patients

In studies that monitor cardiac patients for sero-transformation, a major challenge is the short hospitalization period following surgery. It has been shown that anti-PF4/H antibodies manifest at a median of 6 days post-surgery, but are best detected 14-21 days [68] from initial heparin challenge. Thus, HIT-related serology of cardiac patients is not typically followed to 14-30 days after cardiac surgery without suspicion of HIT as cardiac patients are typically discharged from the hospital within a week if no complications occur. Our study uniquely demonstrates that patients scheduled for CPB surgery that we systematically recruited and tested without suspicion of HIT can produce anti-PF4/H antibody and platelet-activating antibodies. Our study demonstrates that platelet-activating antibody at clinically relevant titres is not predictive of HIT. Six of 38 CPB patients produced platelet-activating antibodies post-cardiac surgery in the SRA, 4 of which induced greater than 50% platelet activation. However, only one of ten of patients that produced platelet activating antibody was identified to have probable HIT. These results suggest that presence of platelet-activating antibody does not cause HIT, such that, SRA positivity independent of consideration of other disease factors does not reflect pathogenicity. Even more surprising was, in one patient (patient no. 58), platelet activating antibodies were present throughout their clinical course. This patient had thrombocytopenia at PO day 2 and 3 but did not develop HIT and had an uneventful postoperative course and was discharged within 5 days post-surgery. These results agree with Nazy et al. suggesting that positive PF4 SRA

results do not indicate risk for HIT. This observation is also corroborated by our results where antibodies causing strong platelet activation in the PF4 SRA do not correspond to platelet activation in the SRA.

5.5 Potency of platelet activation and immunoglobulin class

When we compared immunoglobulin class to platelet activation in the PF4 SRA and the SRA, we found, consistent with previous studies, that all platelet activating sera were anti-PF4/H IgG positive[67]. Additionally, there was a significant relationship between the percentage of platelet activation and presence of PF4/H specific IgA, where PF4 SRA positive samples that were also positive for anti-PF4/H IgA resulted in greatest levels of platelet activation. Previous studies report platelet activating antibody is primarily attributed to IgG, and roles for IgM and IgA anti -PF4/H antibodies in HIT are undefined; therefore, the implications of this finding are unclear. However, the platelet activating function of anti-PF4/H antibodies is directly related to the affinity of the antibody to PF4/H complexes, where anti-PF4/H antibodies with high affinity result in more potent platelet activation[69]. Nguyen et al. propose the affinity of anti-PF4/H antibodies differ in their ability to cluster PF4/H complexes and thus in their pathogenicity; therefore larger PF4/H complexes are more immunogenic[29]. In culmination, the greater the contributions of an antibody in causing or enhancing immune-complex clustering, the greater the resulting platelet activation. In studies that assessed the immune response following immunization with hapten-protein conjugates, average antibody affinity of sera was shown to increase with time. Additionally, progressive increase

in the average affinity of serum antibodies was accompanied by an increased heterogeneity of affinity values. Therefore, if greater antibody affinities develop over time, increased complexity and diversity of antibodies present in the serum may indicate that more affinity maturation cycles have occurred. The IgA anti-PF4/H antibody that is associated with platelet activation does not seem to be correlated with IgA PF4 EIA OD. Therefore, the presence of both IgA and IgG in strongly positive (PF4) SRA patients may reflect an immune response that has gone through several affinity maturation cycles and suggests higher affinity antibodies present in serum. This conjecture needs further investigation to be verified, and we suggest an immediate next step be (PF4) SRA positive sera that have been characterized for immunoglobulin classes should be tested for anti-PF4/H antibody affinity.

5.6 Preoperative immune profile and postoperative production of anti-PF4/H antibodies.

In this study we report for the first time, a preoperative condition that may help to predict postoperative anti-PF4/H IgG antibody. Preoperative anti-PF4/H IgM antibody production found in the supernatant of polyclonally stimulated cells were weakly positively correlated with production of anti-PF4/H antibodies post-surgery. Upon examining the distributions of data with regards to serological and immunological characteristics of CPB sera and preoperative ASC, two populations stand out. The first being, anti-PF4/H IgM antibodies from *in vitro* PBMC are

normally distributed with a positive (right) skew, with 96% of all individuals having a positive IgM PF4 EIA. As opposed to identifying a subgroup of patients who can produce anti-PF4/H IgM antibodies preoperatively, a normal distribution of preoperative *in vitro* IgM production was observed and is potentially due to the natural role for anti-PF4/H antibodies in the primary immune response as suggested by Palankar et al.[55]. Second, almost all patients produce postoperative anti-PF4/H antibody in this patient cohort. Of these, most people that produce anti-PF4/H antibody produce IgG (70%). Therefore, it is perhaps unsurprising that individuals able to produce preoperative anti-PF4/H IgM will have a positive postoperative IgG PF4 EIA result as well. Due to the small sample size, conclusions of the ability for preoperative *in vitro* production of anti-PF4/H IgM antibody to predict post-operative production of IgG cannot be drawn with confidence. However, the transition of IgM to IgG from before surgery to after surgery follows the biological, chronological order, and thus, is a promising lead for future investigations into the HIT-related immune response in CPB patients. The prevalence of IgG seropositive patients after cardiac surgery (previously reported up to 70%), as opposed to the proportion of patients who produce antibody post orthopedic surgery (8-15%)[19] , suggests that characteristics unique to each surgical process may differ in their ability to activate anti-PF4/H antibody-secreting B cells. In particular, cardiac surgery may be a potent memory B cell activator to induce maturation of IgM anti-PF4/H memory B cells into IgG-secreting cells by PO week 3.

5.7 Modelling HIT as a multifactorial disease

HIT prevalence and diagnosis has been studied and reported in detail by many research groups. Namely, heparin type[19,70,71], antibody titre, class[69,72] affinity[73,74], cleavage of and point mutations in FcγRIIA[75,76], the concentration of PF4[71,77], neutrophil activation[78], platelet reactivity to activation[79] have all been studied for pre-test probability or as a way to conclusively determine an individual's likelihood of getting HIT. The current model that best describes the HIT disease is the "iceberg" model described by Warkentin[63]. The iceberg model presents HIT as a disease where clinically pathological consequences are only apparent in a subset of the population, represented by the tip of the iceberg that is visible above the waterline. The model's hierarchy is based on anti-PF4/H seroconversion where those that fall below the waterline seroconvert to sub-clinically relevant threshold levels of anti-PF4/H. Indeed, it has been well established that individuals receiving heparin therapy can readily produce anti-PF4/H antibodies that do not result in thrombocytopenia or thrombosis regardless of previous HIT episodes or exposure to heparin[32]. The iceberg model for HIT does a good job of describing the enigmatic presentation of HIT by representing the large percentage of individuals who demonstrate anti-PF4/H antibody seroconversion but do not result in clinical HIT. Similarly, our study shows that pathogenic antibody that is strongly platelet activating does not directly infer HIT. Given previous work done by research groups that ascribe various disease factors (including but not limited to pathogenic antibody) be relevant in the development of HIT, we would like to suggest an

alternate perspective by modelling HIT as a multifactorial disease. Although this model has not been assigned predictive or pre-test value for diagnosis, it is meant as a method to visualise how various biological and environmental factors could contribute to the development of HIT. This model considers subtle, nuanced factors that affect an individual's likelihood of getting HIT. The strength of this model lies in the ability to easily incorporate future yet-unknown discoveries that may contribute to HIT pathology. It is easy to see that if a degree of pathogenicity were assigned to each factor that contributed to HIT, and each of these conditions could be weighted to represent their ability to drive HIT pathology, it could be used as a diagnostic or predictive model as a clinical tool.

6.0 Study Limitations

In this study, we used a CPB patient cohort to study the production of HIT antibodies. However, although CPB patients have been shown to seroconvert at high frequency, the inferences made in this study may not correlate to disease conditions. Although significance was reached in statistical tests performed on data sets collected from this patient cohort, it is difficult to draw concrete conclusions from the observed correlations. The small sample size means that outliers in the data have greater weight and could lead to correlations that do not carry biological significance. In our dataset comparing preoperative in vitro production of anti-PF4/H antibody and postoperative seroconversion, we observed large confidence intervals suggesting that we cannot be reasonably certain of the degree of correlation between these data sets. In order to draw meaningful, biologically relevant conclusions, the sample size needs to be increased.

Additionally, the recruited patients were all scheduled for CPB surgery. Guidelines for CPB surgery suggest that patients may have heparin-coated catheters inserted prior to surgery date which would constitute as a recent heparin exposure. However, lack of consistent documentation in the electronic charting system which records if patients were catheterized and if heparin was utilized during catheterization made an accurate analysis of heparin exposure prior to CPB surgery challenging to assess.

To quantify pre-operative ASC, we used an ELISPOT assay which is predominantly used to quantify vaccine responses or T cell immune responses. The IgM non-specifically binding to PVDF membrane was not previously documented and lead to our inability to quantitate ASC specifically secreting IgA or IgG anti-PF4/H antibody. Spots formed that were derived from an ambiguous antibody class was problematic as HIT pathogenesis is primarily associated with IgG class antibody and therefore were of great interest to us. Insight into the preoperative immune profile for class-switched memory may have been important for understanding postoperative immune responses. In addition, the high levels of IgM sticking to PVDF membrane lead to high levels of background spots in ELISPOT multititer well plates that did not contain capture antibody. This increased background in the negative control well was especially challenging in ELISPOTs performed with antigen-specific and polyclonal stimulation. Since the frequency of PF4/H specific cells in antigen-stimulated wells was higher, it was also more likely that the non-specific IgM binding to the PVDF membrane was PF4/H specific. To address this, we used an alternate negative control where spots formed in wells without PBMC added were considered background spots.

7.0 Conclusions

In a subset of individuals, *in vitro* stimulated PBMCs can produce IgA, IgG and IgM ASC against PF4/H. The frequency of individuals who have PBMC that can form IgM antibodies is positively skewed but, normally distributed and are in line with studies that suggest these anti-PF4/H IgM antibodies are not pathogenic and may instead have a role as a first line of defence in the immune response. We also demonstrated that in a cohort of patients undergoing CPB surgery, preoperative production of IgM anti-PF4/H antibodies by *in vitro* stimulated PBMC are weakly positively correlated to postoperative IgG anti-PF4/H seroconversion. To our knowledge, this is the first report of a preoperative condition that suggests a postoperative outcome for HIT-related antibodies. This finding is the first step towards understanding how to predict which patients will develop HIT-related antibodies. Furthermore, we investigated CPB patient serology using sera from three time points: pre-surgery, following surgery but before hospital discharge (3-5 days after surgery) and approximately 3 weeks after surgery. Based on PF4/H-dependant EIA results from the PO week 3 period, we found that frequency of seroconversion in this post-cardiac surgery patient cohort to be greater than previously reported (92%). Among these patients, 27% developed antibodies which could activate platelets in a functional assay by PO week 3; 6 of which could activate platelets in the highly specific SRA that is frequently used to confirm clinical HIT. The potency of platelet activation in this patient subset was related to the presence of circulating IgA anti-PF4/H antibodies. Taken together, the serology

of this patient cohort suggests that IgM anti-PF4/H antibodies are present in the general population and do not confer pathogenicity. The positive correlation between preoperative in vitro production of anti-PF4/H IgM antibodies and postoperative IgG seroconversion suggests that IgM memory B cells may undergo maturation and secrete IgG after CPB surgery, a process which may enhance anti-PF4/H antibody production by facilitating an opportunity for immunization and a providing a pro-inflammatory environment. The ability of a HIT-related antibody to be platelet activating has been shown in past studies to relate directly to the affinity for PF4 or PF4/H. Over time, affinity maturation of antibodies allows tighter binding of an antibody to its antigen, however, tracking the maturity of an antibody has been difficult. One method of assessing the maturity of antibody against an antigen suggested is by evaluating the abundance and diversity of antibodies produced in an immune response as the diversity of antibodies is correlated with the presence of high-affinity antibody. Therefore, the presence of IgA anti-PF4/H may discriminate the potent platelet activators from non-potent platelet activators as it is an indicator for antibody diversity. Lastly, we also present 3 case studies of patients. The first of which is the first patient to our knowledge that shows immunological memory for IgG anti-PF4/H antibody. The second is a patient who had platelet activating antibody prior to and throughout the clinical course but did not get HIT. Lastly, we report the pre-operative immune profile and post-operative serology for a patient who developed probable HIT. We note that anti-PF4/H antibodies and other disease factors of HIT are often described as required but not

sufficient to cause HIT. We propose that future studies of HIT should consider HIT as a multifactorial disease that requires several synergistic components to occur in order to have clinical relevance.

8.0 Future Directions

This study reported for the first time, a preoperative condition that may predict a postoperative outcome related to HIT antibodies in cardiac surgery patients. The discovery of this relationship is timely as there are currently a limited number of preventative measures against HIT. The next steps to take in order to understand if the correlation in IgM anti-PF4/H production in the preoperative state to postoperative IgG production is dependent on the medical intervention in order to pose the following questions:

1) does orthopedic surgery, and other cohorts of patients at risk for HIT, show the same correlation as cardiac surgery patients for antibody production?
2) If so, is the barrier for antibody production a surgical procedure or a generically inflammatory environment?
3) If not, what is unique about cardiac surgery conditions that lead to anti-PF4/H antibody production?

Secondly, investigations of what various antibody classes within a serum sample divulge about the affinity of the antibody should be made. An indication of antibody affinity and perhaps platelet-activating capacity would lead to another useful predictor for HIT outcomes.

Lastly, HIT disease factors and well researched individually but there lacks a unifying understanding of the causes and underlying mechanisms for HIT. This study and others highlight the futility of designing research studies for HIT from traditional perspectives of immunization. In future studies, when thinking of and designing experiments to study HIT, it will be important to consider HIT as a multifactorial disease. Continuing to build and understand a model for HIT as a disease will become an invaluable tool in research and clinical applications.

9.0 Figures

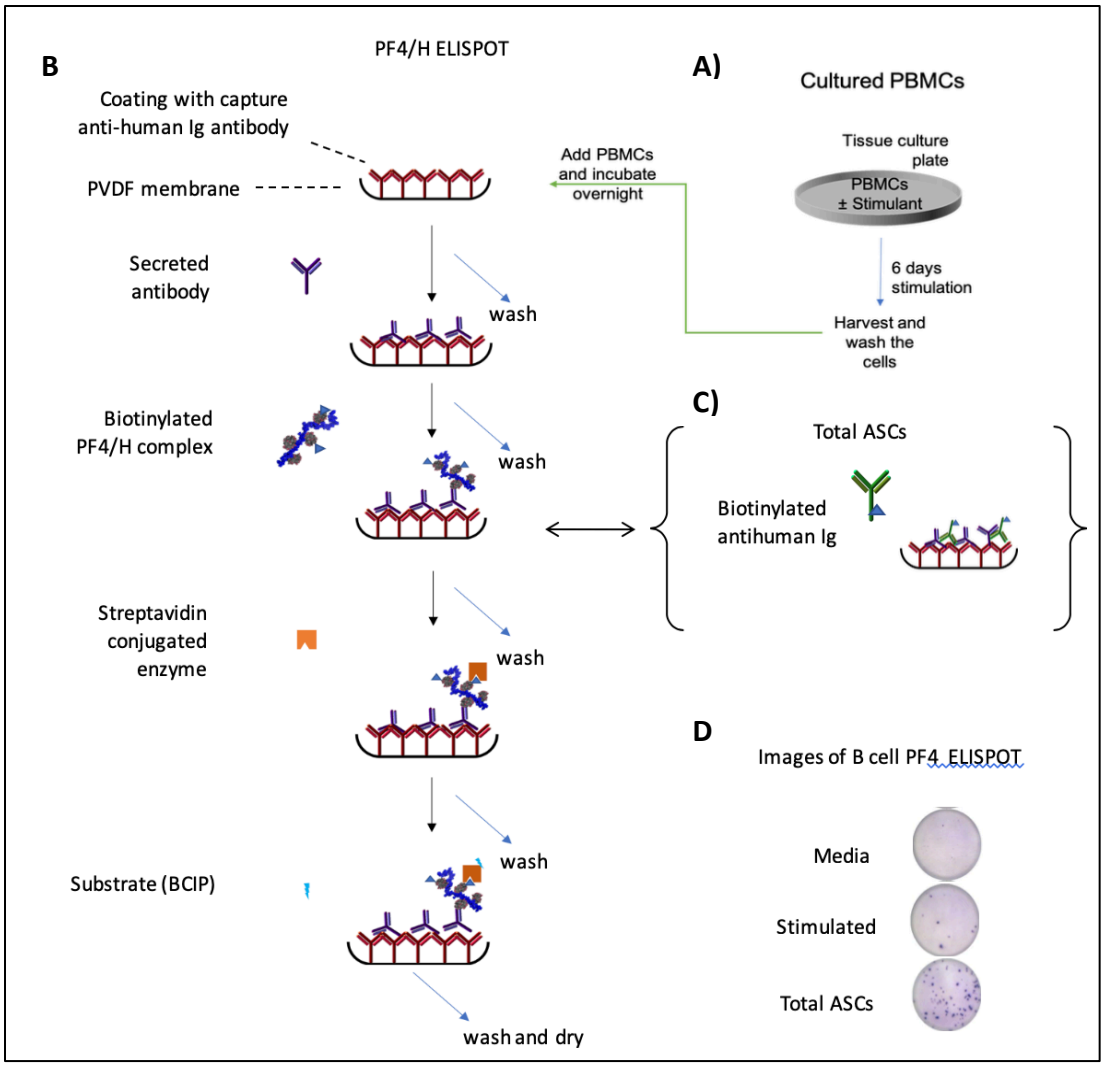
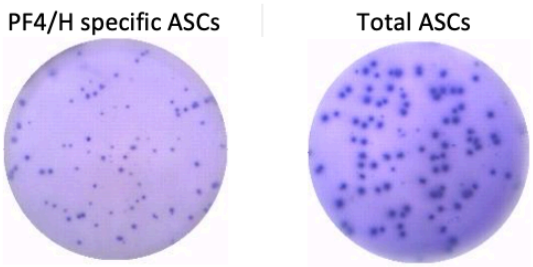


Figure 1. Schematic of PF4/H ELISPOT assay A) PF4/H specific ELISPOT: Pre-activated PBMC were seeded onto pre-coated PVDF multititer plates. B) Human IgA, IgG, or IgM antibodies secreted by ASCs were captured onto the plate; biotinylated PF4/H complexes were captured by antibodies specific for PF4/H. Bound PF4/H complexes were detected using streptavidin-conjugated alkaline phosphatase and its substrate BCIP to form spots representative of ASCs secreting anti-PF4/H specific antibodies. C) biotinylated anti-human IgA, IgG or IgM was used to detect the total number of ASC and detected using streptavidin conjugated alkaline phosphatase and BCIP to form spots representative of ASC secreting IgG, IgM or IgA. D) Representative ELISPOT wells of cells incubated for 6 days with (media) and without pre-activation (stimulated) using polyclonal stimulants detected using biotinylated PF4/H complexes and pre-activated cells detected using biotinylated anti-human IgM antibodies.



Spots per well	97.7	90.3
±SD	20.2	5.5

Figure 2. Quantitative detection of anti-PF4/H hybridoma cells by ELISPOT assay. 100 KKO cells/well were incubated on anti-mouse coated PVDF bottomed 96 well multititer plates, then bound IgG was detected by biotinylated anti-mouse IgG antibody or biotinylated PF4/H complexes and subsequently alkaline phosphatase-conjugated streptavidin. Distinct spots were seen for both methods of detection (either anti-mouse IgG or PF4/H protein complexes).

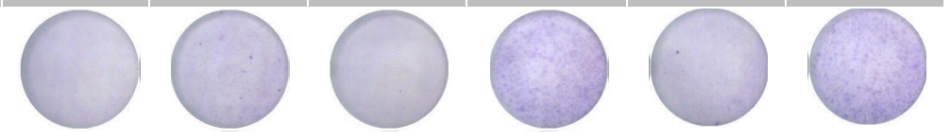
PF4	-	-	+	+	-	+
Heparin	-	-	+	-	+	+
R848 IL2	-	+	-	+	+	+
						
Spots per well	4	42	2.5	213.5	25	200
±SD	4.2	7.1	3.5	16.3	1.4	17.0

Figure 3. Detection of anti-PF4/H antibody-secreting B cells using different in vitro stimulation. PBMC (10^5 cells/ well) from a representative individual was pre-activated with combinations of PF4, heparin, R848, and IL2 as indicated for 5 days. Following [pre-activation, the PBMC were incubated overnight in the ELISPOT plate and number of specific anti-PF4/H IgM antibody-secreting cells was determined.

	Day 1	Day 2	Day 3	Day 4	Day 5
Spots per well	0	0	97	121	358
±SD			15.5	7.07	14.14

Figure 4. Production of antigen-specific spots in PF4/H and polyclonal pre-activated PBMC increases over time. A timeline of spots formed using the PF4/H, R848 and IL2 pre-activated cells in the ELISPOT was used to quantify antigen-specific IgM antibody secreting cells increase over time. PBMC were stimulated using R848, IL2, and PF4/H and were plated after, 1-5 days of pre-activation on an ELISPOT plate and detected using biotinylated PF4/H complexes. Wells shown are from a representative individual.

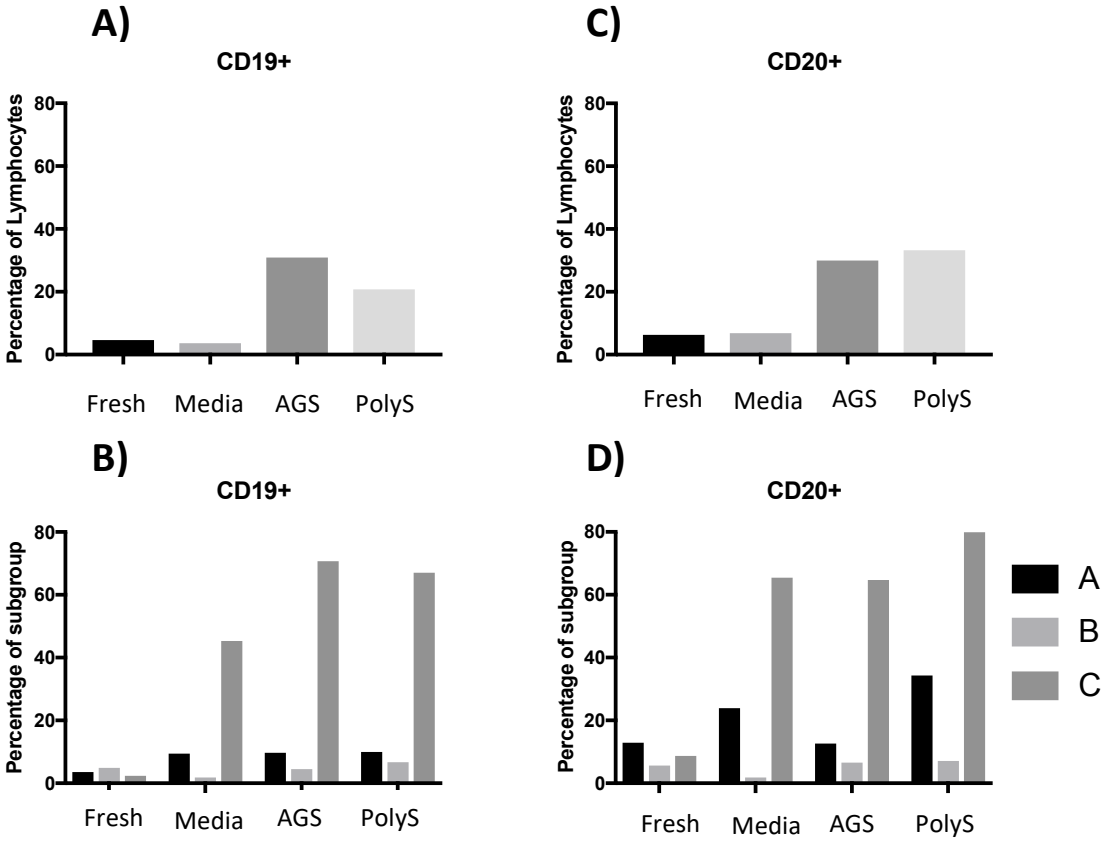


Figure 5. B cell lineage markers increase in stimulated PBMC. The total number of A) CD19+ or C) CD20+ cells prior to stimulation, as well as after 6 days of no stimulation, stimulation with R848 and IL2 (PolyS), or with R848, IL2 and PF4/H (AGS) in media was assessed using flow cytometry and presented as a percentage of total lymphocytes. B) CD19+ and D) CD20+cells in each subgroup of lymphocytes (A, B and C) were assessed and presented as a percentage of subgroup A, B or C.

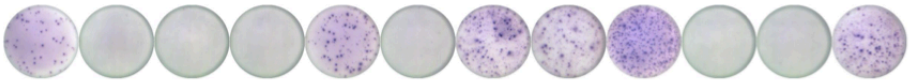
Coat	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	None		
Detection	IgA			IgG			IgM			IgA	IgG	IgM
												
Spots per well	44	0	0.3	0.3	79.9	0	71.8	48.4	270	0.1	0.5	82.8
±SD	8.9	0	0.7	0.5	15	0	10.8	10.6	24.6	0.4	0.8	11.4

Figure 6. Human IgM binds membrane non-discriminately to PVDF membrane. Polyclonally stimulated PBMC from a healthy donor were seeded onto PVDF membrane plates coated with monoclonal anti-human IgM, IgG or IgA in the ELISPOT at 10^5 cells/well. Antibodies were detected using biotinylated anti-human antibodies for class IgA, IgG, and IgM. Spots are detectable when using anti-human IgM antibodies regardless of coating antibody as well as when no coating antibody was used.

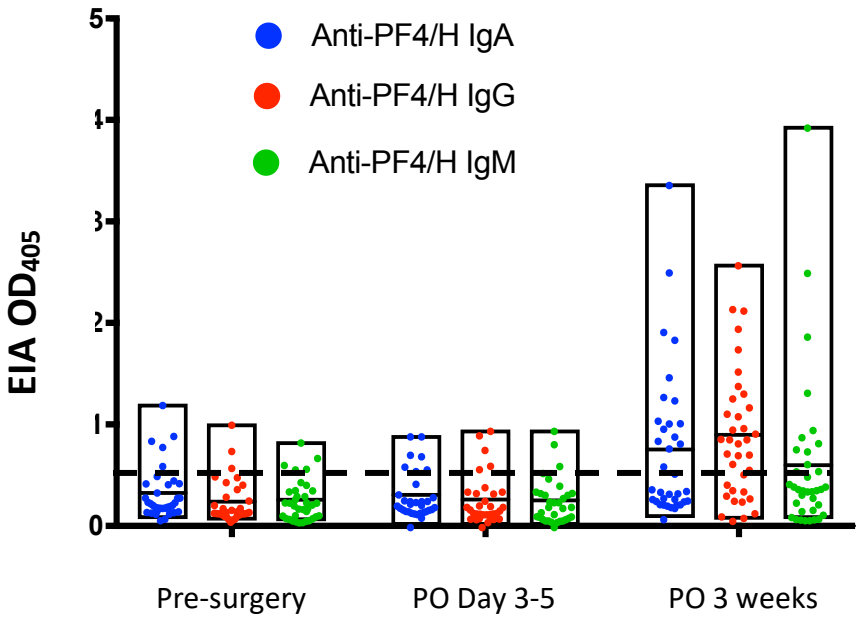


Figure 7. Levels of anti-PF4/H antibodies in CPB patients before and after surgery. CPB patients were followed during their surgery process, a serum sample was taken pre-surgery at the pre-operative consultation (*n*=38), prior to release from hospital 3-5 days after surgery (*n*=33) and 2-3 weeks post-surgery(*n*=38). IgA (blue), IgG (red) and IgM (green) against PF4/H were measured using a class-specific EIA. OD was recorded, where OD ≤0.45 is considered a positive, represented here as a dotted line. (PO = postoperative)

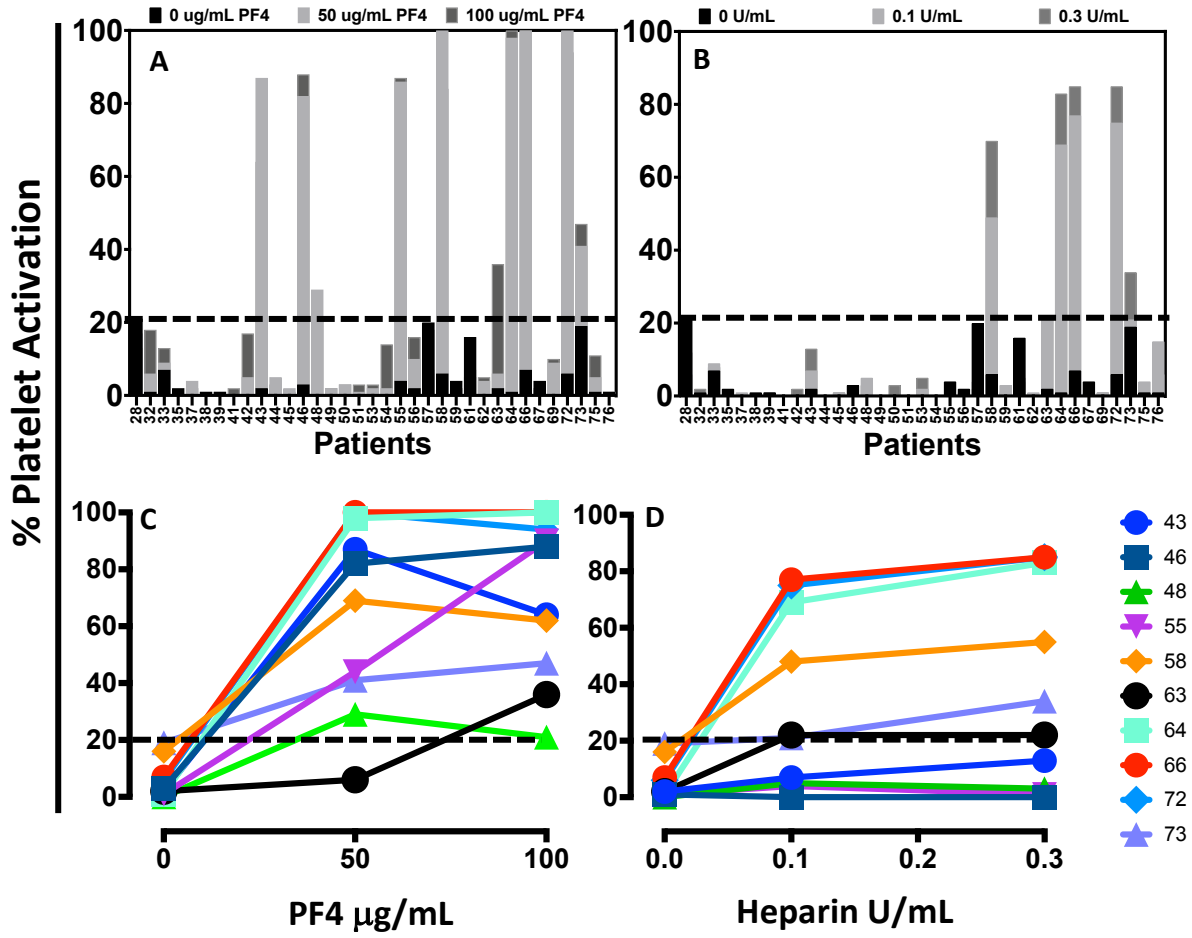


Figure 8. Platelet-activating antibody produced by CPB patients after surgery. Patient sera from post-operative week 3 were screened in the A) PF4 SRA and the B) SRA. In each test, results were categorized as positive if platelet release was greater than 20% when compared to control (dotted line marks 20% release). Each sample was tested using three concentrations of PF4 or heparin, the percentage of release is shown in overlaid histograms. C) shows the platelet release for patients who were positive for the PF4 SRA and D) shows the platelet release for patients who were positive for the SRA.

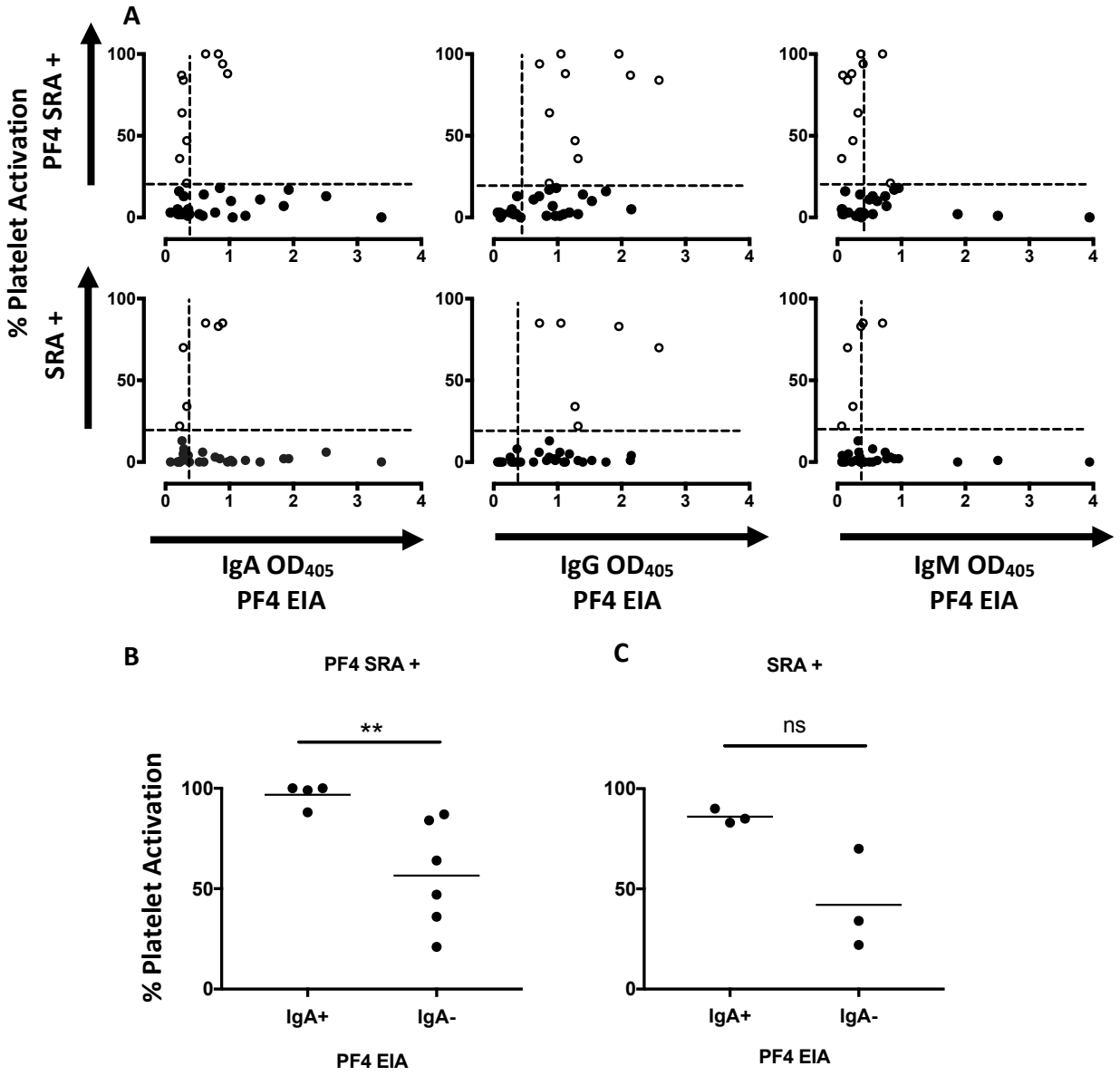


Figure 9. Characterization of postoperative anti-PF4/H antibodies by class and platelet activation. A) Patient sera (*n*=33) at PO week 3 PF4 EIA specific for IgA (column 1), IgG (column 2) or IgM (column 3) (OD [positive >0.45] shown with vertical dotted line) were compared to percentage of platelet activation in the PF4 SRA (row 1) or the SRA (row2) (positive >20% shown with horizontal dotted line). Patients who were positive in the B) PF4 SRA or C) SRA are shown grouped by IgA seropositive (IgA+) or seronegative (IgA-) in the PF4/H dependent EIA. Patients had significantly greater platelet activation when IgA+ than IgA- in the PF4 SRA group (p=0.0095). Differences in platelet activation in IgA+ and IgA- in the SRA+ group were not significant (p=0.10).

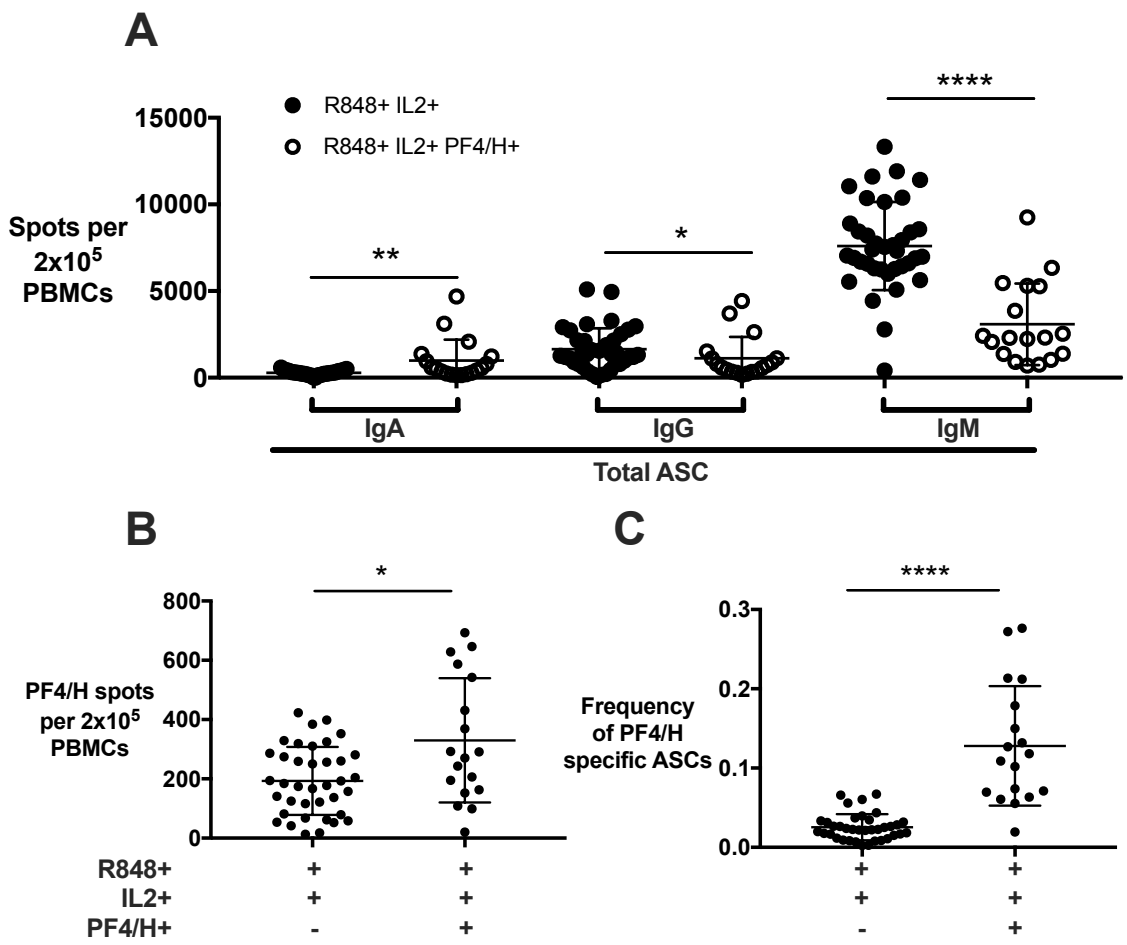


Figure 10. PF4/H antigen-specific and total ASC in preoperative PBMC from CPB patients. A) Total IgA, IgG or IgM antibody-secreting cells were quantified when PBMC from CPB patient's pre-surgery were pre-activated using [R848 and IL2, *n*=37] or [R848, IL2, and PF4/H, *n*=18]. Total ASC were significantly decreased for IgG(p=0.03) and significantly increased for IgA (p=0.006), or IgM(p<0.0001) when pre-activated with antigen (Mann-Whitney test). B) PF4/H specific IgM spots were enumerated and displayed per $2x10^5$ PBMC when pre-activated with or without antigen; pre-activation with antigen significantly increased specific spot formation (p=0.017) (Welch's t-test) C) The frequency of PF4/H specific ASC when pre-activated with antigen is significantly greater than pre-activation without antigen (p<0.0001) (Mann-Whitney test). In R848 and IL2 pre-activated PBMC, PF4/H specific ASC is correlated with total IgM ASC (Spearman'sρ (95%CI)=0.44(0.11,0.68), P=0.009), lastly, in R848, IL2 and PF4/H pre-activated PBMC, PF4/H specific ASC is positively correlated with total IgM ASC (Pearson's r(95%CI)=0.67(0.30,0.87), P=0.002).

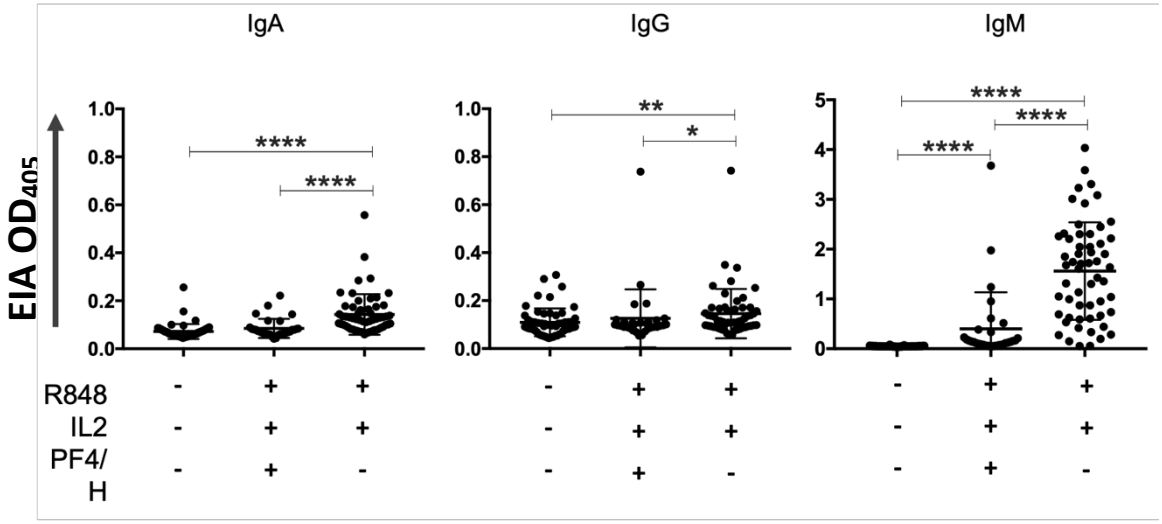


Figure 11. Production of anti-PF4/H antibodies by *in vitro* stimulated PBMC of healthy controls and preoperative CPB patients. PBMC were isolated from peripheral blood from CPB patient pre-surgery (*n*=42) or healthy controls (*n*=15) and pre-activated using R848 and IL2 (*n*=57) or R848, IL2 and PF4/H (*n*=31). Production of class-specific anti-PF4/H antibodies was measured using culture media from pre-activated PBMC were using PF4/H specific EIA. A positive test was identified by OD greater than mean plus two standard deviations of supernatant from PBMC that were cultured for 6 days without pre-activation for each antibody class. Use of different stimulants resulted in significantly different OD for all three population for IgM ($p>0.0001$). For OD of supernatant in the IgA PF4 EIA, PBMC cultured with polyclonal stimulant was significantly different from antigen-stimulated PBMC and PBMC cultured in media alone ($p<0.0001$). The mean OD in the IgG PF4 EIA for PBMC cultured with polyclonal stimulants was slightly greater than when stimulated with antigen stimulants ($p=0.03$) or without stimulants ($p=0.004$) (Mann-Whitney test).

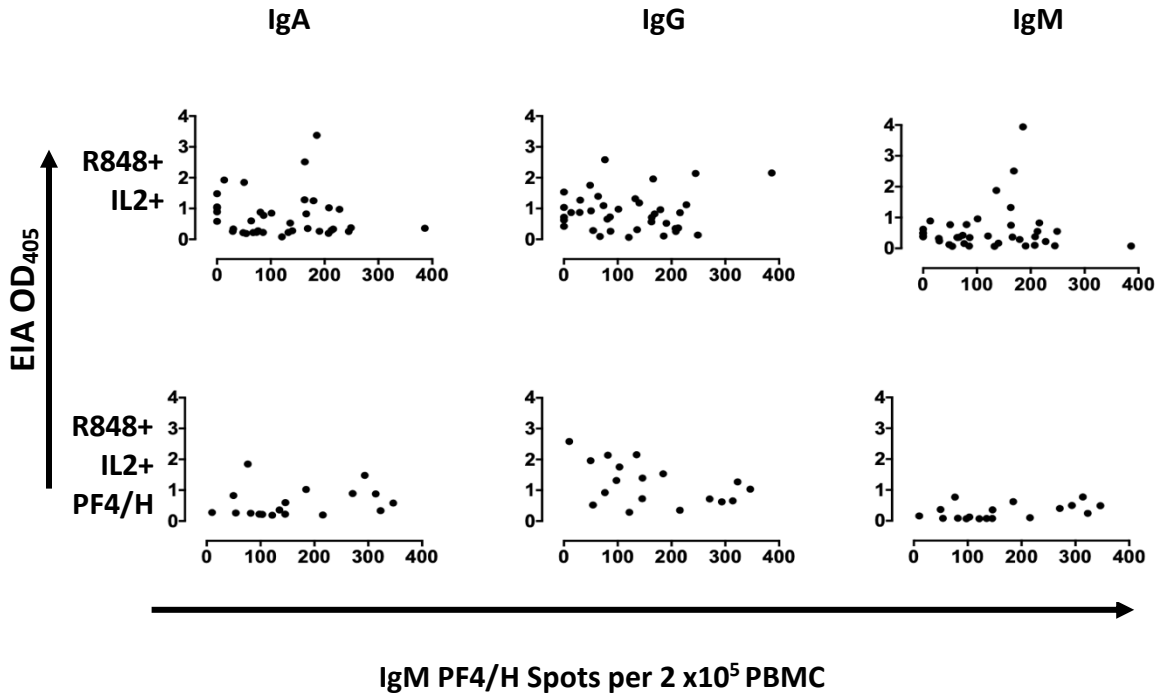


Figure 12. Preoperative PF4/H specific IgM ASC and postoperative anti-PF4/H IgG antibody production in CPB patients. PBMC from CPB patient's before surgery were pre-activated with R848 and IL2 (*n*=37) (row 1) or R848, IL2 and PF4/H (*n*=18)(row 2). Biotinylated PF4/H complexes were used to detect ASC; spots formed, representing IgM ASC, were enumerated and compared to OD of their post-operative (PO week 3) production of anti-PF4/H antibodies using the PF4/H dependent EIA. No significant correlation was found between pre-surgery PF4/H specific IgM ASCs and post-operative seroconversion for either IgA (column 1), IgG (column 2) or IgM (column 3) against PF4/H.

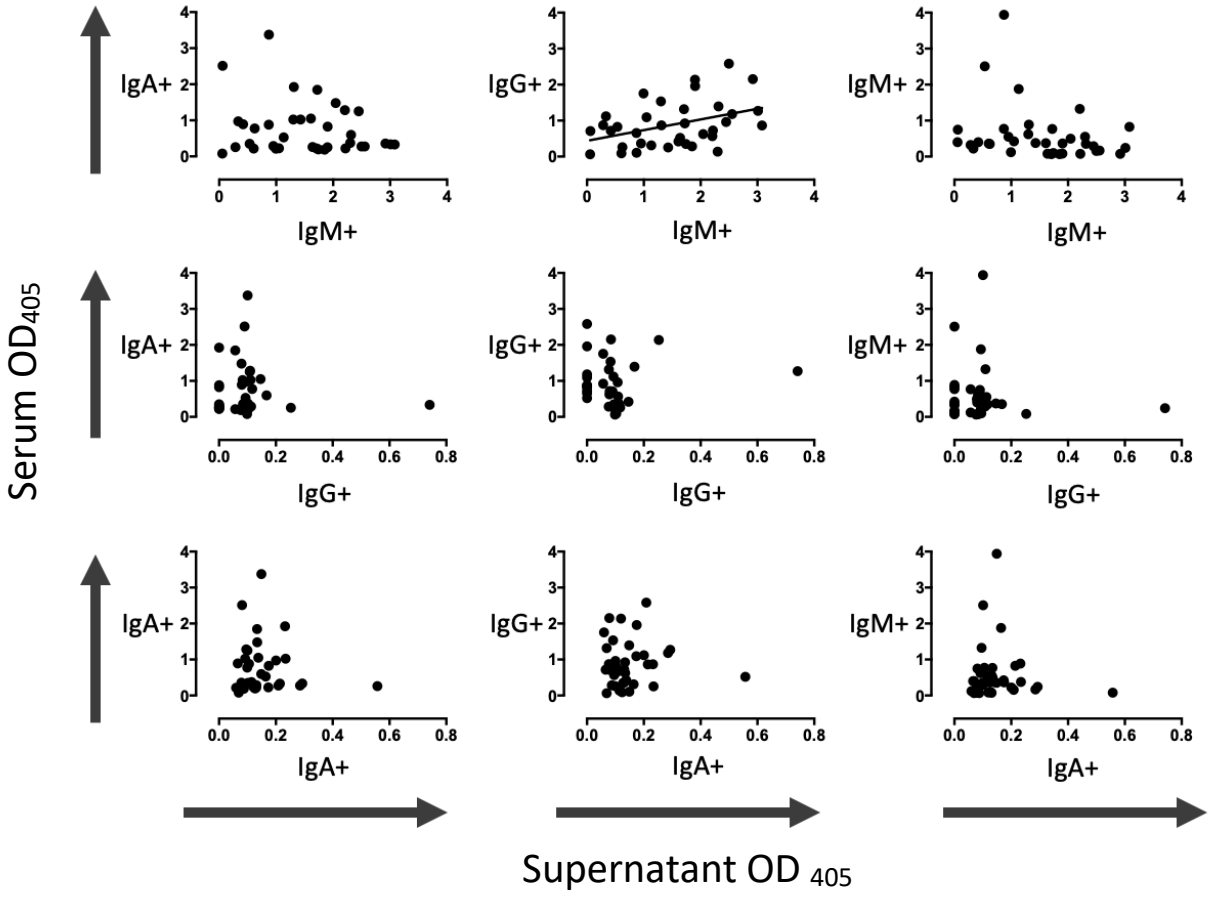


Figure 13. Preoperative production of anti-PF4/H antibodies by *in vitro* stimulated PBMC and postoperative production of anti-PF4/H IgG antibody production. PBMC from CPB patients (*n*=36) were cultured over 6 days with pre-activators R848 and IL2. ODs from PF4/H dependent EIA using supernatant from 6-day cultured PBMC for IgA specific (row 1), IgG specific (row 2) and IgM specific (row 3) PF4/H antibodies were plotted against ODs from CPB patient PO week 3 sera used in PF4/H EIA specific for IgA (column 1,) IgG (column 2) and IgM (column 3). Pre-operative PBMC anti-PF4/H IgM secretion is related to post-operative production of anti-PF4/H IgG antibodies (Spearman's ρ (95% CI)= 0.3928(0.06, 0.65), P=0.018)

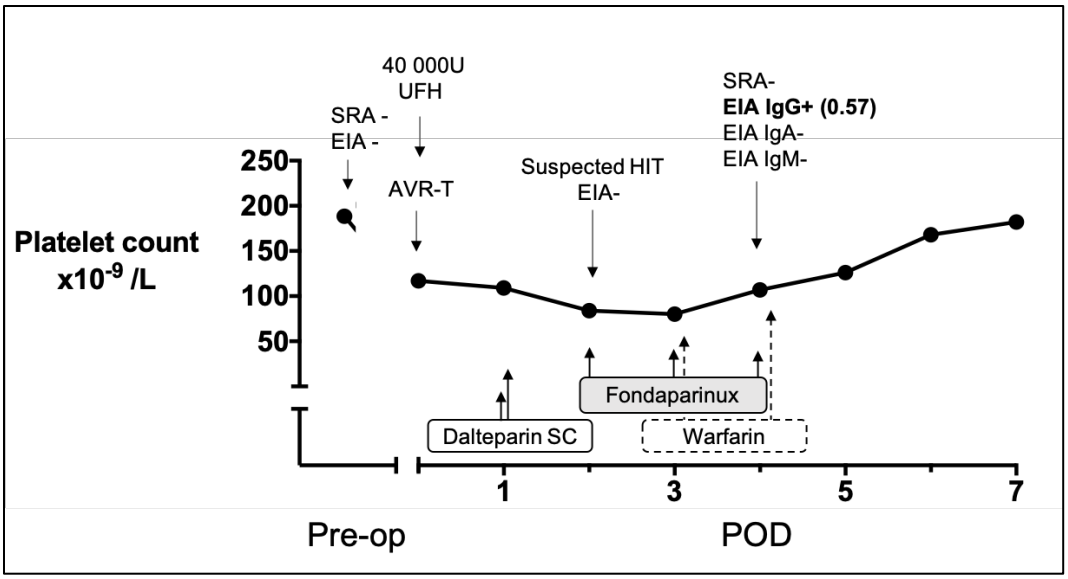


Figure 24. Patient no. 73, case study: Preoperative *in vitro* formation of anti-PF4/H IgG. Pre-surgery, PBMC from peripheral blood was stimulated *in vitro* using B cell activators (R848, IL2, PF4/H). Supernatant from cultured cells contained positive anti-PF4/H IgG (PF4 IgG EIA OD=0.74). Patient was admitted for tissue aortic valve replacement (AVR-T). Patient had platelet count nadir of $142x10^9$/L on surgery day and dropped to the lowest platelet count on POD3 at $80x10^9$/L. The clinician suspected HIT and sent for HIT testing on POD2, but had a negative enzyme immunoassay result (EIA-). Patient was treated with Dalteparin subcutaneously (SC), fondaparinux and warfarin during in-hospital stay. On POD 4, peripheral blood was collected for study, patient was IgG EIA+. During the postoperative period, patient experienced atrial fibrillation and was discontinued on warfarin. On POD21, peripheral blood was collected for study (PO week 3), and found to be PF4 dependent serotonin release and serotonin release assay positive (PF4) SRA+, IgG EIA+, IgA EIA-, and IgM EIA-. This patient has likely had previous surgery related heparin exposure due to a history of bi-lateral knee replacement 7 years prior, although we were unable to confirm use of heparin. Patient did not develop HIT.

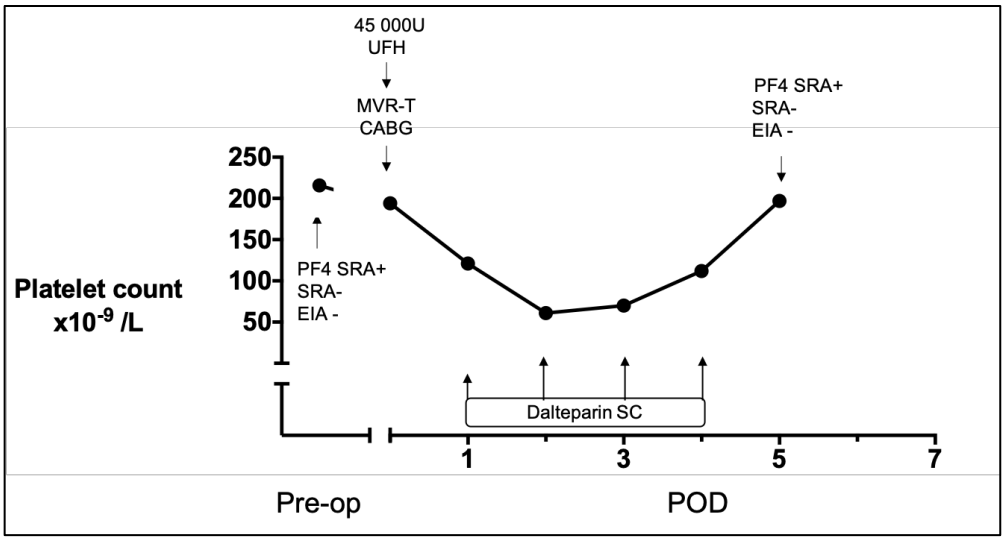


Figure 15. Patient no. 58, case study: Platelet-activating antibody in preoperative and early postoperative periods. Preoperative peripheral blood was tested, and patient tested EIA- (IgG OD= 0.42; IgA OD= 0.22; IgM OD= 0.16) SRA- but PF4 SRA+ (%release (50,100ug/mL PF4) = 4%,46%). The patient had a catheter procedure (unconfirmed use of heparin) pre-operatively and was admitted for tissue mitral valve replacement (MVR-T) with cardiopulmonary bypass graft (CABG). Patient dropped from platelet count 194 to $61x10^9$/L on POD2 but recover platelet count to $197x10^9$/L by POD5. The patient received subcutaneous Dalteparin during POD1-4. On POD5, peripheral blood was collected for study, and patient was found again to be EIA- (IgG OD=0.39; 0.22; 0.13), SRA- and PF4 SRA+(%release (50,100ug/mL PF4) = 9%,58%). The patient was discharged on POD5 uneventfully. On POD34, peripheral blood was collected for study (PO week3) and was found to be EIA+, SRA+, and PF4 SRA+. This patient did not develop HIT.

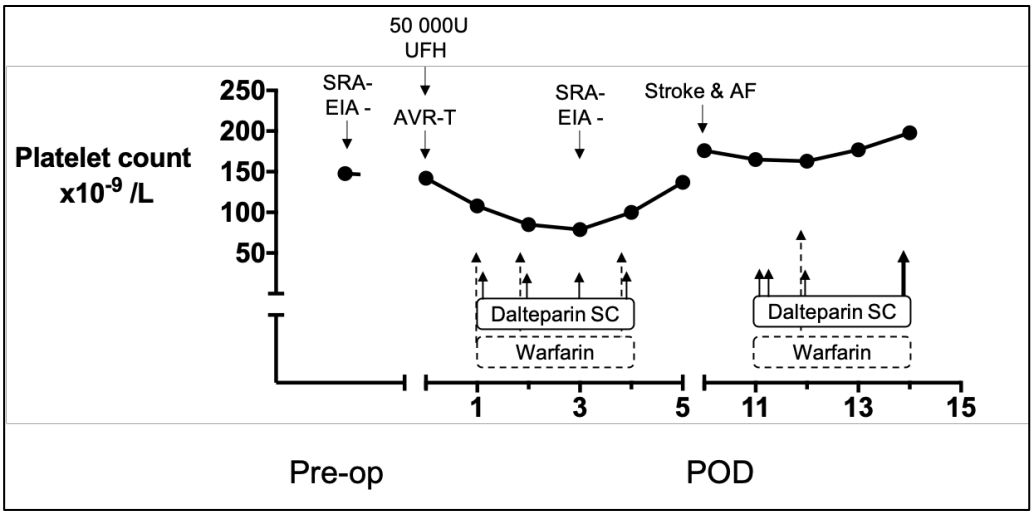


Figure 16. Patient no.64, case study: Probable HIT. Preoperative peripheral blood was collected for study and found EIA-and (PF4) SRA-. The patient was admitted for tissue atrial valve replacement (AVR-T). The patient's platelet count dropped from 142 to 79x10^9/L by POD3, but recovered to 137x10^9/L by POD5. The patient received subcutaneous Dalteparin and warfarin during POD1-4 in-hospital. On POD3, peripheral blood was collected for study, and was found EIA- and (PF4) SRA -. The patient was discharged on POD5, but readmitted on POD10 for atrial fibrillation and stroke. The patient received subcutaneous Dalteparin and warfarin on POD11-14. Platelets did not drop below 150x10^9/L. The patient was discharged on POD15 while continuing warfarin. On POD24 (or 14 days from stroke onset), peripheral blood was collected for study. The patient was found to be EIA+, PF4 SRA+, and SRA+. Patient was retrospectively considered to have probably HIT when the chart was review by a hematologist.

9.1 Supplementary Figures

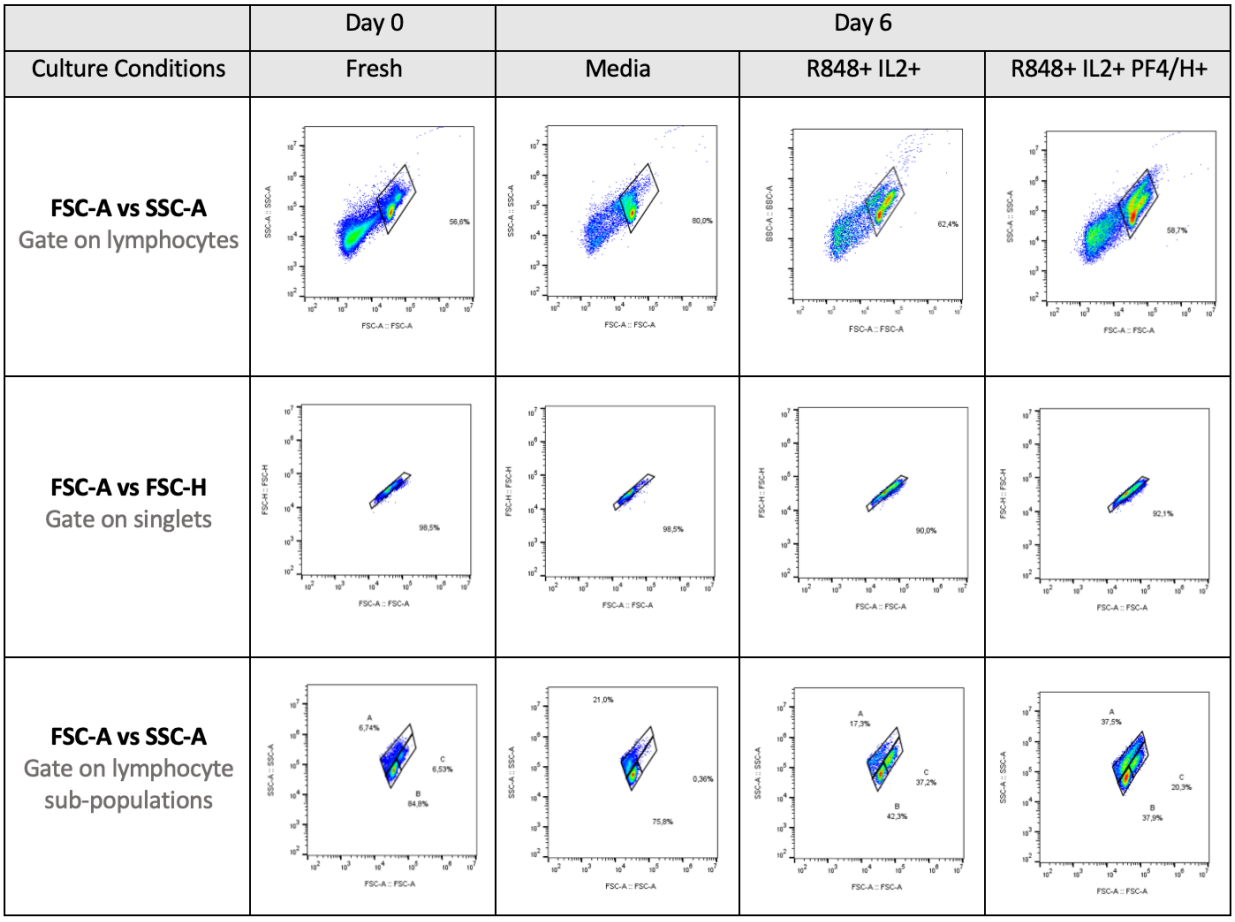


Figure S1. Representative gating strategy for flow cytometry analysis of *in vitro* stimulated PBMC. Single cells were found using FSC-A vs. FSC-H on lymphocytes. The lymphocytes formed three distinct populations and were separated into gates A, B and C and analyzed as for B cells, and T cells individually.

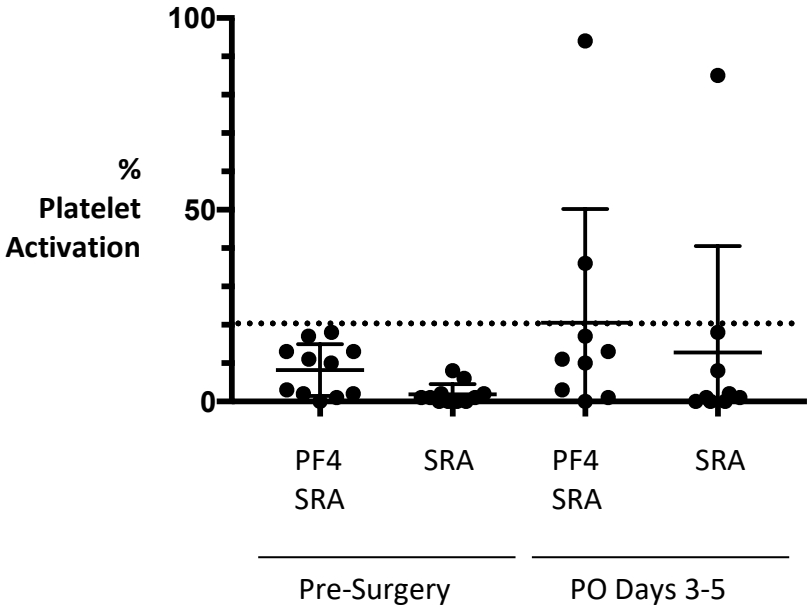


Figure S2. Preoperative or early postoperative anti-PF4/H seropositive CPB patient sera does not predict postoperative production of platelet activating antibody. Patients who were seropositive using PF4/H dependent EIA at the pre-surgical time (*n*=11) or PO days 3-5 (*n*=8) for IgA, IgG, or IgM are shown. The PF4 SRA and SRA were performed at PO week 3 (a positive result is greater than 20%, shown as dotted line).

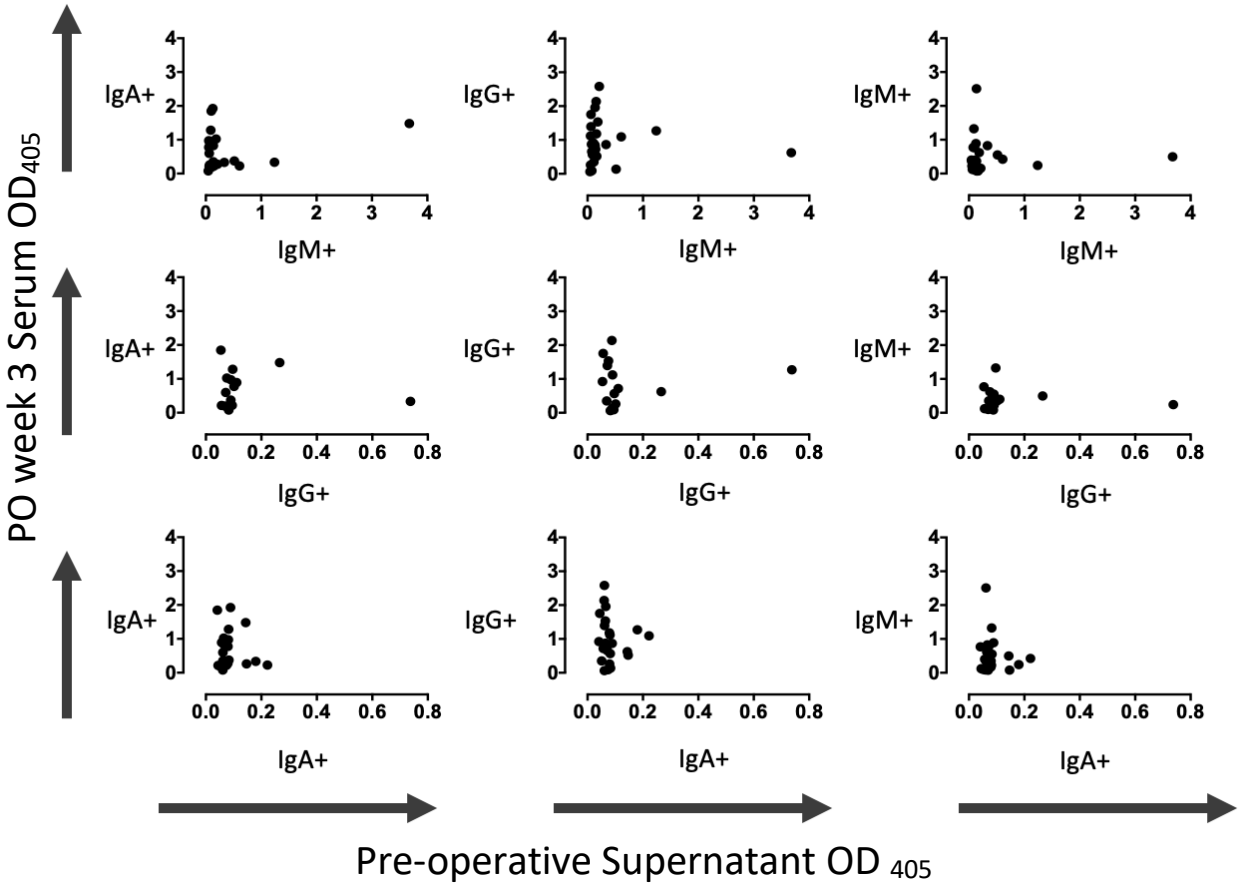


Figure S3. Preoperative PF4/H specific antibody production by antigen-activated PBMC is not related to postoperative production of anti-PF4/H antibodies in CPB patients. PBMC from CPB patients (*n*=18) were cultured over 6 days with pre-activators R848 and IL2. ODs from PF4/H dependent EIA using supernatant from 6-day cultured PBMC with R848, IL2 and PF4/H added for IgA specific (row 1), IgG specific (row 2) and IgM specific (row 3) PF4/H antibodies were plotted against ODs from CPB patient PO week 3 sera used in PF4/H EIA specific for IgA (column 1,) IgG (column 2) and IgM (column 3). No correlations were found.

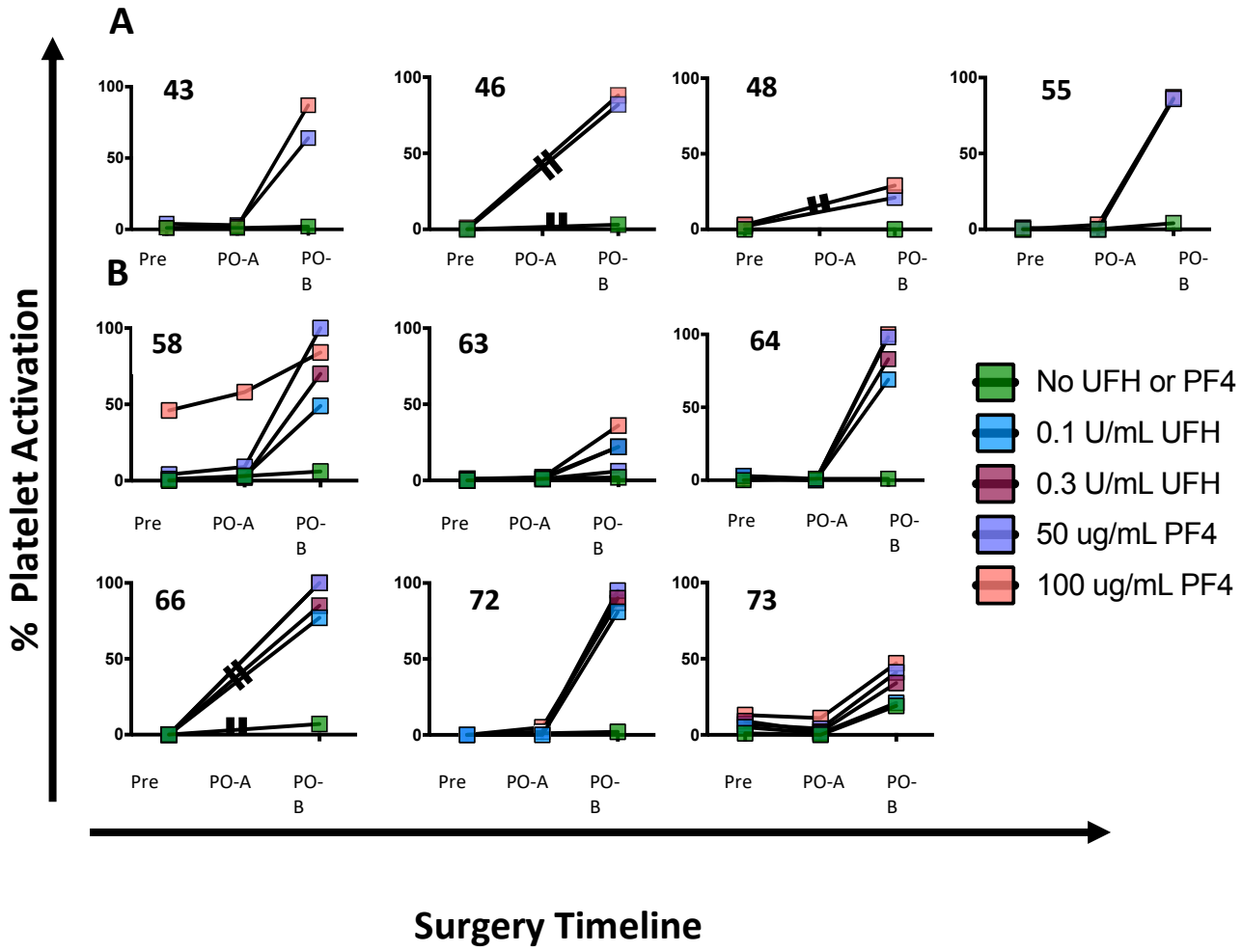


Figure S4. SRA and PF4 SRA of CBP patients pre- and post-surgery. Sera from CPB patients pre-surgery (pre), post-surgery 3-5 days (PO-A) and post-surgery 2-3 weeks (PO-B) was tested in the PF4 SRA and SRA if they were positive in a previous screen (Fig 2a and 2b) A) patients who developed platelet activating antibody detected by the PF4 SRA at PO-B are shown B) patients who developed platelet activating antibody as detected in the SRA at PO-B are shown.

10.0 Tables

Table 1. Characteristics of CPB Patients

No. of patients	38[a]
Age (years)(range)	68 (32-84)
Sex (F/M)	10 : 28

[a] 5 of 38 did not have a serum sample collected prior to discharge

Table 2. Combinations of immunoglobulin classes of anit-PF4/H antibodies formed by CPB patients 3 weeks after surgery

	None	All	IgG Only	IgA Only	IgM Only	IgG and IgA	IgG and IgM	IgA and IgM
n	3	10	11	3	2	5	2	2
%	7.9	26.3	28.9	7.9	5.3	13.2	5.3	5.3

Table 3. Characteristics of CPB patients that produced platelet activating antibody post-cardiac surgery

Characteristic	
Baseline characteristics of patients making platelet activating antibodies	
No. of patients	10
Age (years)(range)	68.7 (57-81)
Sex (F/M)	3 : 7
Preoperative platelet count	204.6[a] (117-257)
Type of Surgery	
Valve surgery	5
CABG + valve surgery	5
Cardiopulmonary bypass time (minutes)	113.96 (38-181)
Cross-clamp time (minutes)	98.39 (29-163)
Anti-PF4 antibody characteristics	
PF4-heparin IgG EIA (OD) (range)[b]	1.5 (0.86-2.58)
Positive PF4 SRA (% release ≥ 20), n (% of total CPB)	4
Positive PF4 SRA and SRA (% release ≥ 20), n (% of total CPB)	6

[a]one patient had mild viral-associated thrombocytopenia pre-surgery
[b]EIA performed on sample drawn at post-operative 3-week time

10.1 Supplementary Tables

Table S1. Lymphocyte subgroups in *in vitro* stimulated PBMC of a healthy donor

	Fresh		Media		R848 IL2		R848 IL2 PF4/H	
	count	%	count	%	count	%	count	%
Events total	63302	n/a	20570	n/a	10623	n/a	27016	n/a
Lymphocytes	35862	57	16457	80	6627	62	15862	59
Single cells	35336	99	16214	99	5966	90	14611	92
A	2382	7	3405	21	1032	17	5479	38
B	29965	85	12290	76	2524	42	5538	38
C	2307	7	58	0	2219	37	2966	20
Viability		90		70		82		70

Table S2. B cell populations found in *in vitro* stimulated PBMC

Stimulant		CD	count	% of subgroup	% of lymphocytes
Fresh	A[a]	38	410	17.2	1.2
	B	27	11387	38.0	32.2
		27,38	10008	33.4	28.3
		38	3746	12.5	10.6
		19,20,21,38[b]	**596**	**2.0**	**1.7**
		19,20,38	**375**	**1.3**	**1.1**
		19,20,21	**240**	**0.8**	**0.7**
		19,20	**132**	**0.4**	**0.4**
	C	38	1756	76.1	4.9
Media	A	**20**	**456**	**13.4**	**2.8**
		38	388	11.4	2.4
		19,20	**273**	**8.0**	**1.7**
		20,38	**100**	**2.9**	**0.6**
		3,8	233	6.8	1.4
	B	27	7313	59.5	45.1
		27,38	2667	21.7	16.4
		38	446	3.6	2.8
		3,4	7989	65	49.3
		3,8	2716	22.1	16.8
	C	n/a	n/a	n/a	n/a
Polyclonal Stimulants (R848 & IL2)	A	**20**	**221**	**21.4**	**3.7**
		38	114	11	1.9
	B	27	770	30.5	12.9
		38	500	19.8	8.4
		27,38	273	10.8	4.6
	C	**19,20,38**	**870**	**39.2**	**14.5**
		19,20	**484**	**21.8**	**8.1**
		20,38	**251**	**11.3**	**4.2**
		38	199	8.6	3.3
Antigen Stimulants (R848, IL2 & PF4/H)	A	38	1540	28.1	10.5
		20	**282**	**5.2**	**1.9**
		19,38	**205**	**3.7**	**1.4**
		20,38	**201**	**3.7**	**1.4**
		19	**120**	**2.2**	**0.8**
	B	38	1811	32.7	12.3
		27	1168	21.1	8.0
		27,38	593	10.7	4.0
	C	**19,20,38**	**1350**	**45.5**	**9.2**
		38	445	15.0	3.0
		19,20	**291**	**9.8**	**2.0**
		19,38	**201**	**6.8**	**1.4**
		20,38	**108**	**3.6**	**1.4**
		19,20,27,38	**104**	**3.5**	**0.7**

[a] A, B and C in this column denote lymphocyte subgroups described in Table S1.
[b] Cell populations that contain B cell lineage markers are bolded

Table S3. T cell populations found in *in vitro* stimulated PBMC

		CD	count	% of subgroup	% of lymphocytes
Media	A[a]	3,8	233	6.8	1.4
	B	3,4	7989	65	49.3
		3,8	2716	22.1	16.8
	C		n/a	n/a	n/a
Polyclonal Stimulants (R848 & IL2)	A		n/a	n/a	n/a
	B	3,4	1564	62.0	26.2
		3,8	608	24.1	10.2
	C	3,4	134	6.0	2.2
		3,8	145	6.6	2.4
Antigen Stimulants (R848, IL2 & PF4/H)	A	3,8	189	3.5	1.3
	B	3,4	3372	60.9	23.0
		3,8	1412	25.5	9.7
	C	3,4	252	8.5	1.7
		3,8	248	8.4	1.7

[a] A, B and C in this column denote lymphocyte subgroups described in Table S1.

Table S4. Trial ELISPOT conditions used for minimizing IgM non-specific background binding

	Conditions tested	IgM total ASC spots in wells with no capture antibody
Wash buffer	PBS TBS NaCl	✓
Membrane	PVDF Nitrocellulose	✓
Antibody	Mabtech Jackson Immunology	✓
Membrane Hydration	30% EtOH 70% EtOH	✓
Incubation of Activated PBMC with PVDF membrane	2h 4h Overnight	✓
Development time with streptavidin-alkaline phosphatase	30min 45min 1hr	✓

Table S5. Supernatant PF4 EIA for pre-CPB patients and healthy donors[a]

	IgA			IgG			IgM		
	PolyS[b]	AgS[c]	NS[d]	PolyS	AgS	NS	PolyS	AgS	NS
n	57	31	57	57	31	57	57	31	57
mean	0.14	0.08	0.07	0.15	0.13	0.11	1.56	0.40	0.05
±SD	0.08	0.04	0.03	0.10	0.12	0.06	0.98	0.73	0.01
Cutoff[e]	0.13			0.23			0.07		
EIA+ [f]	25	4	2	6	2	3	55	26	3

[a] Preoperative patients and healthy donors are combined in this analysis as these populations are not different prior to cardiac surgery
[b] Supernatant from PBMC stimulated using polyclonal stimulants R848 and IL2
[c] Supernatant from PBMC stimulated using antigen and polyclonal stimulants (R848, IL2 and PF4/H)
[d] Supernatant from PBMC cultured in media without stimulants
[e] Cutoff value determined using the mean plus two standard deviations of the OD values from supernatant of PBMC culture in media
[f] number of supernatant samples positive in the PF4 EIA using the established cutoff

Table S6a. Case study: Immune profile of CPB patient pre and post cardiac surgery

Patient no. 73				
	Sample Type	IgA	IgG	IgM
PF4/H EIA				
Pre-surgery	Serum	0.07	0.05	0.05
PO 3-5 days	Serum	0.26	0.57	0.19
PO 3 weeks	serum	0.33	1.27	0.24
Pre-surgery	Supernatant (R848+IL2+)	0.29	0.74	3.00
Pre-surgery	Supernatant (R848+IL2+PF4/H+)	0.18	0.74	1.24
PF4 SRA				
PF4 μg/mL		*0*	*50*	*100*
Pre-surgery	Serum	1	7	13
PO 3-5 days	Serum	0	4	11
PO 3 weeks	Serum	19	41	47
Pre-surgery	Supernatant (R848+IL2+)	0	0	0
Pre-surgery	Supernatant (R848+IL2+PF4/H+)	0	0	0
SRA				
Heparin U/mL		*0*	*0.1*	*0.3*
Pre-surgery	Serum	1	5	9
PO 3-5 days	Serum	0	1	2
PO 3 weeks	Serum	19	21	34
Pre-surgery	Supernatant (R848+IL2+)	0	0	0
Pre-surgery	Supernatant (R848+IL2+PF4/H+)	0	0	0
PF4/H ELISPOT				
		Pre	PO 3 weeks	
IgM PF4/H specific spot / $2x10^5$ PBMC	R848+ IL2+ activated PBMC	59	49	
IgM PF4/H specific spot / $2x10^5$ PBMC	R848+IL2+PF4/H+ Activated PBMC	626	486	

Table S6b. Case study: immune profile of CPB patient pre and post cardiac surgery

Patient no. 58				
	Sample Type	IgA	IgG	IgM
PF4/H EIA				
Pre-surgery	Serum	0.22	0.42	0.16
PO 3-5 days	Serum	0.22	0.39	0.13
PO 3 weeks	serum	0.28	2.58	0.16
Pre-surgery	Supernatant (R848+IL2+)	0.21	0.14	2.50
Pre-surgery	Supernatant (R848+IL2+PF4/H+)	0.07	0.11	0.21
PF4 SRA				
PF4 μg/mL		*0*	*50*	*100*
Pre-surgery	Serum	0	4	46
PO 3-5 days	Serum	3	9	58
PO 3 weeks	Serum	6	100	84
SRA				
Heparin U/mL		*0*	*0.1*	*0.3*
Pre-surgery	Serum	0	1	1
PO 3-5 days	Serum	1	0	0
PO 3 weeks	Serum	1	49	70
PF4/H ELISPOT				
IgM PF4/H specific spot / $2x10^5$ PBMC	R848+ IL2+ Activated PBMC		82	
IgM PF4/H specific spot / $2x10^5$ PBMC	R848+IL2+PF4/H+ Activated PBMC		20	

Table S6c. Case study: immune profile of CPB patient pre and post cardiac surgery

Patient no. 64				
	Sample Type	IgA	IgG	IgM
PF4/H EIA				
Pre-surgery	Serum	0.29	0.16	0.18
PO 3-5 days	Serum	0.25	0.13	0.11
PO 3 weeks	serum	0.83	1.96	0.37
Pre-surgery	Supernatant (R848+IL2+)	0.18	0.17	1.90
Pre-surgery	Supernatant (R848+IL2+PF4/H+)	0.66	0.19	0.14
PF4 SRA				
PF4 ug/mL		*0*	*50*	*100*
Pre-surgery	Serum	0	3	1
PO 3-5 days	Serum	1	0	0
PO 3 weeks	Serum	1	98	100
SRA				
Heparin U/mL		*0*	*0.1*	*0.3*
Pre-surgery	Serum	0	3	3
PO 3-5 days	Serum	1	0	1
PO 3 weeks	Serum	1	69	83
PF4/H ELISPOT				
IgM PF4/H specific spot / $2x10^5$ PBMC	R848+ IL2+ Activated PBMC		178	
IgM PF4/H specific spot / $2x10^5$ PBMC	R848+IL2+PF4/H+ Activated PBMC		100	

11.0 References

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