AUTOREACTIVE B CELLS IN IMMUNE THROMBOCYTOPENIA

AUTOANTIBODIES AND AUTOREACTIVE B CELLS IN THE BONE MARROW AND THE PERIPHERAL BLOOD OF PATIENTS WITH IMMUNE THROMBOCYTOPENIA

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TITLE: Autoantibodies and autoreactive B cells in the bone marrow and the peripheral blood of patients with immune thrombocytopenia

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ABSTRACT:

Introduction: Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by a platelet count less than 100 x 10^9/L. Platelet-bound autoantibodies are detected in the peripheral blood of only 40-50% of ITP patients. The subset of ITP patients who do not have detectable autoantibodies may truly be seropositive, but autoantibodies may not be detected due to limitations of the assays. Studies have also suggested that autoantibodies might be sequestered in the bone marrow where autoantibodies may impair platelet production. In addition, detecting autoreactive antibody-secreting B cells using the Enzyme-linked Immunospot (ELISPOT) assay was shown to be highly sensitive. In this study, the bone marrow and the peripheral blood compartments of ITP patients were tested for the presence of anti-GPIIbIIIa and anti-GPIbIX IgG autoantibodies and autoreactive B cells.

Methods: Bone marrow aspirate and peripheral blood samples were collected from ITP patients (n=12), non-immune thrombocytopenic patient controls (n=3), and healthy controls (n=5). Mononuclear cells were isolated and tested for the presence of anti-GPIIbIIIa and anti-GPIbIX IgG autoreactive B cells before stimulation and after stimulation with R848 and IL-2 using the ELISPOT assay. Anti-GPIIbIIIa and anti-GPIbIX IgG autoantibodies were detected in the bone marrow and the peripheral blood using the direct antigen capture assay.

Results: In our study, we detected autoantibodies and autoreactive B cells of known specificity in the bone marrow of a subset of ITP patients. Detecting anti-GPIIbIIIa and

anti-GPIbIX autoantibodies in the bone marrow or the peripheral blood had a sensitivity of 42% and testing both compartments increased the sensitivity to 58%, while maintaining 100% specificity. Autoreactive B cells were detected at low frequencies with low specificity in the bone marrow and the peripheral blood of a subset of ITP patients. The majority of the ITP patients without detectable autoantibodies in the peripheral blood did not have autoantibodies in the bone marrow, and autoreactive B cells were not detected in either compartment.

Conclusion: Examining both the bone marrow and the peripheral blood compartments for autoantibodies or autoreactive B cells increased the sensitivity. Furthermore, detecting autoantibodies using the antigen capture assay is more sensitive and specific than detecting autoreactive B cells using the ELISPOT assay.

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LIST OF ABBREVIATIONS:

AC	Antigen capture
ACD	Acid citrate dextrose
AMR	Ashwell-Morell receptors
AP	Alkaline phosphatase
ASCs	Antibody-secreting cells
BCA	Bicinchoninic acid assay
BCIP	5-bromo-4-chloro-3'-indoylphosphate
BCR	B cell receptor
BMMCs	Bone marrow mononuclear cells
BSA	Bovine serum albumin
BSS	Bernard Soulier Syndrome
CBC	Complete blood count
CD	Cluster of differentiation
DMSO	Dimethyl sulfoxide
Dsg3	Desmoglein 3
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked Immunospot
FBS	Fetal bovine serum
FVIII	Factor VIII
GP	Glycoprotein
GT	Glanzmann thrombasthenia
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ig	Immunoglobulin
IL	Interleukin

ITP	Immune thrombocytopenia
IVIg	Intravenous immunoglobulins
MAIPA	Monoclonal antibody-specific immobilization of platelet antigen
MPS	Mononuclear phagocyte system
NBT	Nitro-blue tetrazolium chloride
O.D.	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PRP	Platelet-rich plasma
PVDF	Polyvinylidene fluoride
RA	Rheumatoid arthritis
RPMI	Roswell Park Memorial Institute medium
R848	Resiquimod
SD	Standard deviation
SDS	Sodium dodecyl-sulfate
SHM	Somatic hypermutation
SLE	Systemic lupus erythematosus
TBS	Tris buffer saline
TLR	Toll-like receptor
TPO	Thrombopoietin
WGA	Wheat germ agglutinin

DECLARATION OF ACADEMIC ACHIEVEMENT

Sabrina Shrestha performed all of the experiments described in this thesis under the supervision of Dr. Ishac Nazy and Dr. Donald M. Arnold with the exception of the following:

- Dr. Peter Horsewood and Sabrina Shrestha purified platelet glycoproteins.
- James Smith and Hina Bhakta conducted the direct antigen capture assay to detect autoantibodies in the bone marrow aspirate and the peripheral blood samples.
- Jennifer Cui and Sabrina Shrestha conducted experiments involved in optimization of the Enzyme-linked Immunospot assay to reduce non-specific binding of IgM antibodies.

1.0 INTRODUCTION:

1.1 Platelets

1.1.1 Production

Platelets are small, disc-shaped, anucleate blood cells produced in the bone marrow[1]. The bone marrow is a specialized niche that supports the development of various types of blood cells through highly regulated mechanisms. One such mechanism is megakaryopoiesis, a developmental process of hematopoietic stem cells into progenitor cells that eventually gives rise to megakaryocytes[2]. As the megakaryocytes mature, the cells grow and express various maturation proteins while undergoing endomitosis. Endomitosis occurs when the cell increases its nuclear content without cell division and causes the cell ploidy to increase up to 128N in megakaryocytes. As invaginations form on the membrane of the megakaryocytes, proplatelet extensions develop and release platelets into the vascular sinusoids of the bone marrow[3]. This phenomenon is called thrombopoiesis, the process of platelet production. Platelets inherit the cytoplasmic contents of megakaryocytes that contain proteins necessary for platelet function and survival. In healthy individuals, a single megakaryocyte gives rise to 1000-3000 platelets and approximately 10^{11} platelets are produced daily, resulting in a peripheral blood platelet count of 150-450 x $10^9/L$[2]. Platelets typically survive in circulation for 7-10 days and are removed from the system due to senescence by either apoptosis or phagocytosis by macrophages and hepatocytes[1].

1.1.2 Function

Platelets are primarily known for their role in hemostasis and thrombosis[4]. Hemostasis is a physiological process that regulates the balance of clot formation and clot lysis. Upon injury of the blood vasculature, platelets activate and adhere to the endothelial cells of the vessel wall. These platelets secrete proteins and aggregate using membrane receptors such as glycoprotein IIbIIIa (GPIIbIIIa) and glycoprotein IbIX (GPIbIX), which is necessary to form a platelet plug and prevent blood loss at the site of injury[4]. The abnormal number and/or functional capabilities of platelets can result in hematological disorders associated with uncontrolled bleeding or excessive clotting. For example, Glanzmann thrombasthenia (GT) and Bernard-Soulier syndrome (BSS) are both inherited bleeding disorders caused by quantitative and/or qualitative defects of the platelet membrane receptors, GPIIbIIIa and GPIbIX, respectively[5]. A steady rate of platelet turnover and platelet survival are also essential for maintaining a normal platelet count. A low platelet count can result in thrombocytopenia and increase the risk of bleeding, whereas, a high platelet count can result in thrombocythemia and increase the risk of clotting[6,7]. Aside from hemostasis, platelets are an important component of the immune system since platelets interact with various immune cells and secrete immunomodulatory proteins involved in innate and adaptive immunity[8]. Furthermore, platelets can also be targeted by the immune system in some diseases associated with induced immunity and autoimmunity[9,10].

1.2 Immune Thrombocytopenia

1.2.1 Definition, Classification and Clinical Diagnosis

Immune thrombocytopenia (ITP) is an acquired autoimmune bleeding disorder characterized exclusively based on a platelet count less than 100 x 10^9/L and an increased risk of bleeding[11]. ITP is further classified into primary or secondary ITP, based on the association of thrombocytopenia with an underlying cause. Primary ITP consists of 80% of the cases where patients have isolated thrombocytopenia with an unknown etiology[11]. The remaining 20% of the cases are secondary ITP since thrombocytopenia is either induced by drugs or is related to infections or diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), HIV and *Helicobacter pylori* infections[12].

ITP affects both children and adults, but the course of the disease varies between the two age groups[13]. ITP in children tends to be acute and usually requires minimal intervention due to the self-limiting nature of the disease. Whereas, ITP in adults is usually chronic and involves various types of interventions. ITP has an incidence rate of 3.3/10^5 adults every year, and a prevalence rate of 9.5/10^5 adults, as females are affected slightly more than males[14]. Not all ITP patients are symptomatic, but those who are, display a wide range of bleeding manifestations[11]. These symptoms include mucocutaneous bleeding such as petechiae and purpura, spontaneous bruising, and increased frequency of bleeding events such as nasal, oral and gastrointestinal bleeding[11,15]. In extreme cases, 1.5% of ITP patients experience intracranial hemorrhages[11]. Symptoms usually appear when the platelet count is <30 x 10^9/L and severe bleeding tend to occur at a platelet count <10 x 10^9/L[16]. Clinical

studies assessing platelet survival and bleeding in thrombocytopenic patients have also estimated that a minimum platelet count of 7-8 x 10^9/L is required to maintain hemostasis[17,18]. Multiple ITP patients with the same platelet count can display symptoms of varying severity. Although there is a lack of correlation between the platelet count and the risk of bleeding, the risk of bleeding is known to increase with age[19]. Once patients are diagnosed with ITP, they are classified as newly diagnosed for the first 3 months, persistent between 3-12 months, and chronic for those with ITP for more than 12 months[11]. Certain cases are also classified as severe ITP when patients present bleeding symptoms that require immediate treatment.

1.3. Pathophysiology of ITP

1.3.1. Autoantibody-Mediated Platelet Destruction

The initial breakthrough in the field of ITP occurred in 1951 when Dr. Harrington conducted the Harrington-Hollingsworth experiment[20]. He infused blood from ITP patients into himself along with several healthy individuals and observed as the platelet counts declined rapidly to severe thrombocytopenic levels[20]. Once the platelet counts increased back to normal, Dr. Harrington hypothesized that the thrombocytopenia-causing agent must be a plasma factor. Following this proposition, autologous and allogeneic platelets were shown to absorb plasma factors from the IgG rich fraction of the ITP patient's plasma[21]. These findings indicated that the plasma factors were platelet-specific IgG autoantibodies. The majority of the autoantibodies detected in the peripheral blood of ITP patients are IgG and have specificity for either GPIIbIIIa or GPIbIX[22,23]. A single platelet expresses

approximately 80 000 copies of GPIIbIIIa and 50 000 copies of GPIbIX, which makes these glycoproteins the two most abundant complexes that the autoantibodies can bind to on the platelet surface[24]. Additionally, ITP patients also had a reduced platelet survival rate[25]. Since these findings, autoantibodies have become the hallmark of ITP, and autoantibody-mediated platelet destruction is thought to be the underlying mechanism of the disease[26]. Once ITP patients produce autoantibodies, platelets are opsonized and targeted for destruction through the Fc receptors expressed on the phagocytic cells of the mononuclear phagocyte system (MPS) (Figure 1a)[27]. Phagocytic cells such as macrophages internalize and degrade platelets and present platelet peptides on the cell surface. Further interactions with autoreactive T cells and B cells result in the production of autoantibodies[28]. The spleen is one of the compartments of the MPS and is considered to be the primary site of platelet destruction since this is where macrophages reside, where B cells produce autoantibodies, and where 1/3rd of the individual's platelets are sequestered[29,30]. A recent study has suggested that platelet destruction can also occur through an Fc independent mechanism[31]. Hepatocytes can phagocytose platelets that are opsonized with anti-GPIbIX autoantibodies through the Ashwell-Morell receptors (AMR). Additionally, anti-GPIIbIIIa and anti-GPIbIX autoantibodies have also shown to induce complement activation, resulting in platelet destruction through the classical complement pathway[32]. Whether a specific autoantibody-mediated mechanism is involved in certain subsets of ITP patients or if multiple mechanisms are involved, all of these mechanisms result in accelerated platelet destruction.

1.3.2 Autoantibody-Mediated Platelet Underproduction

Aside from platelet destruction, studies have also suggested that autoantibodies might be involved in platelet underproduction in the bone marrow. This was initially indicated by radiolabelled platelet survival studies that showed a reduced rate of platelet turnover in ITP patients compared to healthy individuals[33–36]. Further examination of bone marrow biopsies showed that the megakaryocyte count was either normal or increased in ITP patients. The increase in megakaryocytes could be a result of the body attempting to compensate for the low platelet count and/or maturation of megakaryocytes may have stopped at an early stage of development[37]. The majority of megakaryocytes in ITP patients did not have characteristics of maturation, rather cytoplasmic degeneration and apoptotic features were observed[38]. Inhibition of apoptosis in megakaryocytes has been suggested to prevent platelet production. However, the involvement of apoptosis during platelet production is debatable. Furthermore, the presence of GPIIbIIIa and GPIbIX on the surface of megakaryocytes along with the detection of an increased amount of IgG antibodies on the surface of ITP patient's megakaryocytes suggests that autoantibodies are present in the bone marrow[39,40]. Furthermore, in vivo studies showed that antibodies produced by ITP patient's splenocytes could bind to megakaryocytes, but antibodies produced by healthy individuals could not[39]. The abnormal characteristics of megakaryocytes observed in the bone marrow biopsies of ITP patients were also observed in megakaryocytes that were cultured in ITP patient's plasma[38]. More specifically, anti-GPIbIX autoantibodies were shown to prevent megakaryocyte differentiation and reduce the formation of proplatelet extensions, which is necessary for platelet production[38]. In a recent study, ITP patient's

plasma containing anti-GPIIbIIIa, anti-GPIbIX, and anti-GPIaIIa antibodies was shown to interfere with the interactions between megakaryocytes and the bone marrow niche[41]. These findings contribute to the hypothesis that autoantibodies likely interfere with platelet production in the bone marrow[40].

The bone marrow is not only a niche where megakaryocytes develop and produce platelets, but it is also a major site of antibody production[42]. This is where long-lived plasma cells reside and are known to actively secrete antibodies throughout an individual's lifetime as part of the humoral memory. If anti-GPIIbIIIa and anti-GPIbIX antibody-secreting cells (ASCs) are present in the bone marrow, the secreted autoantibodies can bind to megakaryocytes and interfere with megakaryopoiesis and/or thrombopoiesis, impairing platelet production. The bone marrow is also a compartment of the MPS where phagocytic cells reside. Phagocytosis of megakaryocytes by monocytes and neutrophils were observed in the bone marrow biopsies of some ITP patients[38,43]. Similar to the spleen, the bone marrow is a compartment that contains ASCs (long-lived plasma cells), the target cells of interest (megakaryocytes and platelets), and the cells that are capable of destroying the target cells (phagocytes).

1.4 Treatment

Treatment regimens for ITP patients aim to stabilize the platelet count to clinically safe levels while also minimizing the potential toxicity associated with therapy[11,15]. Patients receive treatment only if they have bleeding symptoms and/or their platelet count is <30 x 10^9/L. The treatment options attempt to increase the platelet count by reducing the rate of

platelet destruction and/or enhancing the rate of platelet production. Thus, response to specific treatment options provides insight into the potential mechanisms involved in ITP.

The first line of therapy consists of administering immunosuppressive agents including corticosteroids such as prednisone and dexamethasone[44]. However, only 10-30% of the patients undergo long-term remission[11]. Immunomodulatory agents such as IVIg and anti-D globulin are usually used in severe cases to increase the platelet count rapidly. The presence of additional antibodies saturates the Fcγ receptors on the macrophages and reduces the rate of platelet destruction[45].

Once patients fail to respond to first-line therapy, they receive second-line therapy[11]. These treatment options include Rituximab, an anti-CD20 monoclonal antibody used to deplete CD20 expressing B cells by apoptosis, complement-mediated lysis, and/or antibody-dependent cytotoxicity[46,47]. Patients who were administered rituximab had low frequencies of CD20 expressing B cells, but plasmablasts and plasma cells, which are the only types of B cells that secrete antibodies were present in the spleen[48,49]. This is because plasma cells do not express CD20 and likely explains why some patients do not respond to Rituximab[48]. Additionally, the presence of autoreactive plasma cells have been speculated to exist in the bone marrow and also contribute to autoantibodies in the peripheral circulation[50]. This is especially suggested by patients who have residual autoantibodies after splenectomy since the major site of autoantibody production does not exist. Splenectomy has been the gold standard treatment option for ITP patients due to the high remission rate (60-70%) but is usually not recommended due to the invasive nature of the procedure[51,52]. One of the recently developed therapies includes thrombopoietin (TPO)

mimetics such as romiplostim and eltrombopag[53]. TPO mimetics bind to the TPO receptors (cMpl) expressed on the surface of megakaryocytes. This promotes proliferation and differentiation of megakaryocytes, enhancing platelet production.

A staircase model is recommended for managing ITP patients as treatment options with minimal toxicity are prioritized[11]. However, most patients are administered a wide range of options since ITP is diagnosed based on the exclusion of other disease and treatment becomes more of a trial and error approach. Patient response to treatment options that target autoantibody production and platelet destruction such as immunosuppressants, IVIg, splenectomy, anti-D, and Rituximab all indicate the important role of autoantibody-mediated platelet destruction in ITP. However, patients with a lack of response to therapies such as Rituximab and splenectomy further suggest the involvement of the bone marrow compartment in the pathophysiology of ITP since this is another major site of antibody production. Thus, an in-depth understanding of the mechanisms involved in the disease is necessary to develop individualized treatment plans, improve patient outcomes and minimize excessive use of unnecessary drugs.

1.5 Laboratory Assays

1.5.1 Detecting Autoantibodies

Over the years, various serological assays have been developed to detect platelet-specific autoantibodies in ITP patients. The lack of sensitivity and/or specificity of these assays has prevented its use as a diagnostic tool[54]. For example, platelet-associated IgG (PAIgG) assays detect IgG autoantibodies bound to the surface of the platelets[55]. Although

over 90% of the ITP patients had an increased level of autoantibodies on the platelets, the diagnostic specificity was low, since non-immune thrombocytopenic patients also had platelet-bound autoantibodies[54,56]. Platelet antigen-specific assays such as the monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay and the antigen capture (AC) assay can detect platelet-bound autoantibodies (direct) and unbound autoantibodies present in circulation (indirect)[54,57]. Although these assays have high specificity, the diagnostic sensitivity is low since only 40-50% of ITP patients have detectable platelet-bound autoantibodies specific to GPIIbIIIa and/or GPIbIX[58,22]. Detecting free circulating autoantibodies (indirect assay) had an even lower sensitivity[59]. Furthermore, including autoantibodies with specificity to other antigens such as glycoprotein GPIaIIa (GPIaIIa) and glycoprotein IV (GPIV) did not increase the diagnostic sensitivity since these autoantibodies are usually detected alongside anti-GPIIbIIIa and/or anti-GPIbIX autoantibodies[60,61]. Furthermore, IgA and IgM autoantibodies have also been detected, but at low frequencies and are thought to have a minor role in the pathogenesis of ITP[62,63]. Although diagnostic assays are not available for clinical use, the presence of platelet-specific autoantibodies indicates that the patient has ITP, whereas the lack of autoantibody detection does not rule out the disease.

1.5.2. Limitations of Detecting Autoantibodies

Autoantibody testing assays are not only technically demanding, but several factors involved in these assays also limit their use. Detecting platelet-bound autoantibodies are limited by the low number of platelets in the circulation of ITP patients since opsonized platelets are destroyed rapidly[26]. Detection of unbound autoantibodies is limited since

platelets quickly sequester most of the autoantibodies in circulation[64]. The ELISA based assays such as the AC assay also uses monoclonal antibodies that bind to the platelet antigens[59]. Some of the autoantibodies bound to certain epitopes on the antigen could sterically hinder the binding of monoclonal autoantibodies to the antigen, limiting the detection of the autoantibodies in the assay. Thus, the question remains whether the subset of ITP patients without detectable autoantibodies are truly seronegative or if these assays lack sensitivity. Rather than detecting autoantibodies themselves, examining autoreactive ASCs as a biomarker may have a higher diagnostic sensitivity.

1.5.3. Detecting Autoreactive ASCs

The presence of autoantibodies in ITP patients indicates that the autoreactive cells that secrete these autoantibodies must also be present. The B cell Enzyme-linked Immunospot (ELISPOT) assay can be used to quantify autoreactive ASCs by examining the ability of an individual to produce autoantibodies. The ELISPOT assay uses the antigen of interest to detect antigen-specific antibodies secreted by a single ASC within a heterogeneous pool of cells[65]. A single ASC secretes antibodies forming a foci/spot with a high density of antibodies in the center that gradually diffuses towards the edges of the spot. Kuwana et al. and Chen et al. have used the ELISPOT assay to detect autoreactive ASCs in the peripheral blood of ITP patients and have shown that detecting autoreactive ASCs is diagnostically more sensitive than detecting autoantibodies[66–69]. Detecting anti-GPIIbIIIa ASCs in ITP patients had a diagnostic sensitivity of 86% and specificity of 83%, whereas detection of only anti-GPIbIX ASCs had a diagnostic sensitivity of 43% and

specificity of 89%[66]. Altogether, detecting both anti-GPIIbIIIa and anti-GPIbIX ASCs increased the sensitivity to 90% while maintaining high specificity.

1.6 B Cells

1.6.1. Development, Tolerance Mechanisms and Autoimmunity

B cells are an important component of the immune system because they produce antibodies to neutralize foreign pathogens while tolerating self-antigens[70]. B cells are produced from hematopoietic stem cells in the bone marrow, and as the B cells develop, the cells encounter various positive and negative selection processes[71]. Once the B cells pass the early stages of development (pro-B cell and pre-B cell), immature B cells express a wide range of IgM B cell receptors (BCR) on the cell surface. Approximately 75% of these immature B cells are either reactive to self-antigens or are polyspecific, meaning the cells can react to both self-antigens and foreign antigens[72]. The immature B cells with high affinity to autoantigens either undergo receptor editing to produce BCRs that are not reactive to self-antigens or undergo clonal deletion through apoptosis. However, some of the autoreactive B cells with a medium-low affinity for autoantigens or the polyspecific B cells may escape central tolerance in the bone marrow and enter peripheral circulation[73]. In the peripheral blood of healthy individuals, 2.5% of leukocytes are B cells, of which 64% are naïve B cells, 31% are memory B cells, 5.4% are immature B cells, and 2.1% are plasma cells and plasmablasts[9]. The cells that enter peripheral circulation from the bone marrow are known as naïve B cells and also express IgM antibodies. When the autoreactive B cells that express high-affinity BCRs are detected in the periphery, the B cells are eliminated by

either apoptosis, anergy or receptor editing. The naïve B cells are activated when the autoantigen binds to the BCR and causes the cell to proliferate and differentiate into plasma cells that secrete low-affinity autoantibodies. The memory B cells also have antigen specificity and can be directly activated with the antigen (T cell-independent) or through the interactions with T cells (T cell-dependent), causing the memory B cells to proliferate and terminally differentiate into plasmablasts and plasma cells[42]. The plasmablasts become plasma cells, which are short-lived antibody-secreting cells. During this process, more memory B cells are also produced and are present in circulation. Memory B cells tend to have receptors with high antigen binding affinity since these cells have undergone somatic hypermutations (SHM) and possibly immunoglobulin class switch recombination[42]. Some of the plasmablasts also migrate to the bone marrow and become long-lived plasma cells known to secrete antibodies for several decades. This is where the long-term survival of plasma cells is supported, making the bone marrow one of the important antibody secreting sites[74,75].

The production of pathogenic autoantibodies is an indication of immune dysregulation involving autoreactive B cells[76]. The existence of autoreactive cells is the result of the evasion of immune tolerance mechanisms that the B cells encounter at multiple stages of development. The two main checkpoints for eliminating autoreactive B cells are the central tolerance checkpoint in the bone marrow and the peripheral tolerance checkpoint in the peripheral blood[77]. Autoreactive B cells not only contribute to autoimmunity by secreting autoantibodies but also present self-antigens to T cells due to their antigen presentation capabilities[78]. The autoreactive B cells can also secrete proinflammatory

cytokines amplifying the autoimmune response. Along with ITP, diseases such as SLE, rheumatoid arthritis and type I diabetes are examples of autoimmune diseases that involve the production of autoantibodies[79–81].

2.0 RESEARCH OUTLINE:

2.1. Overall Objective:

The main objective of this study was to detect and compare the presence of anti-GPIIbIIIa and anti-GPIbIX autoantibodies and autoreactive B cells in the bone marrow and the peripheral blood compartments of ITP patients.

2.2. Rationale:

Platelet-specific autoantibodies are a hallmark of ITP. However, autoantibodies are detected in the peripheral blood of only 40-50% of ITP patients. Autoantibodies may not be detected in the remaining 50-60% of the patients due to the low sensitivity of the assays used to detected autoantibodies, or the patients are truly seronegative. Rather than detecting autoantibodies in ITP patients, studies have shown that detecting autoreactive ASCs is diagnostically more sensitive[66,67]. However, if some patients are truly seronegative, there must be other mechanisms aside from autoantibody-mediated platelet destruction involved in ITP. Autoantibody-mediated platelet underproduction has been proposed as another mechanism since studies have shown that ITP patients have low platelet turnover, megakaryocytes express platelet-specific antigens (GPIIbIIIa and GPIbIX), and an elevated amount of IgG antibodies have been detected on the surface of megakaryocytes from ITP patients[33–36]. Additionally, in vitro studies have shown that autoantibodies from ITP patients can bind to megakaryocytes and interfere with platelet production. Autoantibodies may not be present in circulation because the autoantibodies are sequestered in the bone marrow. The bone marrow is also a compartment where long-lived plasma cells reside and

actively secrete antibodies[74]. If anti-GPIIbIIIa and/or anti-GPIbIX autoreactive ASCs are present in the bone marrow, autoantibodies can bind to megakaryocytes and impair megakaryopoiesis and/or thrombopoiesis, reducing platelet production (Figure1b). The bone marrow also contains phagocytic cells such as macrophages, which are capable of destroying opsonized cells such as megakaryocytes and/or newly produced platelets. Furthermore, autoantibodies produced in the bone marrow could be a source of autoantibodies found in the peripheral circulation, as suggested by the patients who have residual autoantibodies after splenectomy. Assessment of autoantibodies and autoreactive ASCs in the bone marrow and the peripheral blood compartments of ITP patients is a novel approach to understand the mechanisms involved in ITP.

2.3. Hypothesis:

Platelet-specific autoantibodies and autoreactive B cells are present in the bone marrow compartment of ITP patients.

2.4. Specific Objectives:

1. Optimize an ELISPOT assay to quantify anti-GPIIbIIIa and anti-GPIbIX autoreactive ASCs.
2. Quantify autoreactive ASCs and detect autoantibodies in the peripheral blood of ITP patients, non-immune thrombocytopenic controls, and healthy controls.
3. Quantify autoreactive ASCs and detect autoantibodies in the bone marrow of ITP patients, non-immune thrombocytopenic controls, and healthy controls.

4. Categorize ITP patients into subsets depending on the presence and absence of autoantibodies and/or autoreactive ASCs in the bone marrow and the peripheral blood.

3.0 MATERIALS AND METHODS:

3.1. Subjects and Sample Collection

Peripheral blood and bone marrow aspirate samples were collected from patients and healthy donors for experimental purposes. All participants provided informed consent in accordance with the Hamilton Integrated Research Ethics Board for the study: A Prospective Longitudinal Study to Assess Long-Term Safety of Treatments and the Epidemiology of Bleeding in Immune Thrombocytopenia (McMaster ITP Registry Project).

Peripheral blood samples (20-90 mL) were collected from patients and healthy controls in sterile vacutainers containing acid citrate dextrose (ACD) by phlebotomists. Sets of bone marrow aspirate and peripheral blood samples were collected from chronic ITP patients (n=12), non-immune thrombocytopenic patient controls (n=3), and healthy controls (n=5) at the Boris Clinic in the McMaster University Medical Center. Bone marrow aspirate samples (9 mL) were collected from the posterior iliac crest in heparin (1 mL) by Dr. Donald Arnold. Peripheral blood samples (30 mL) were also collected in ACD from the bone marrow aspirate donors. Experimental processing of the samples was initiated within 2 hours of sample collection.

3.2. GPIIbIIIa Platelet Lysate Preparation and Purification

GPIIbIIIa was affinity-purified from freshly outdated human platelets [within 10 days from the expiration date of the platelet-rich plasma (PRP)] as described in a previous study[82]. PRP was pooled and centrifuged at 350 x *g* for 15 minutes to remove contaminating

red blood cells (RBCs) and then centrifuged at 2400 x *g* for 45 minutes to pellet platelets. Platelets were washed once with the wash buffer with EDTA (20 mM Tris HCl, 150 mM NaCl and 1 mM EDTA at pH 7.5) and twice with the wash buffer without EDTA. Platelets were centrifuged at 2400 x *g* for 45 minutes. The platelet pellets were weighed and resuspended in lysing buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM $CaCl_2$, 0.02% NaN_3, 0.1% Triton X-100 and EDTA free protease inhibitors at pH 7.5) at a ratio of 1 g of platelets/1 mL of lysing buffer. Platelets were lysed overnight at 4°C and stored at -80°C until further use.

Platelet lysates were thawed and centrifuged at 30 000 x *g* for 1 hour, and solubilized platelet lysates were obtained. Concanavalin A beads were washed with buffer A (20 mM Tris HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 0.02% NaN_3 at pH 7.5) and mixed with solubilized platelet lysate for 2 hours at room temperature. The beads and the supernatant were poured in a column and washed with the wash buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 0.02% NaN_3 and 100 mM mannose at pH 7.5). Proteins bound to the beads were eluted with the elution buffer (20 mM Tris HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 0.02% NaN_3 and 100 mM methylmannoside at pH 7.5). The elution fractions were collected, and absorbances were measured at 280 nm using the Eppendorf BioPhotometer. The elution fractions were tested on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions to analyze the protein contents.

3.3. GPIbIX Platelet Lysate Preparation and Purification

GPIbIX was affinity purified from freshly outdated human platelets (within 10 days from the expiration date of the PRP) as described previously[83]. PRP was pooled and centrifuged at 350 x *g* for 15 minutes to remove contaminating RBCs and then centrifuged at 2400 x *g* for 45 minutes to pellet platelets. Platelets were washed 3 times with the wash buffer (120 mM NaCl, 30 mM D-glucose, 30 mM sodium citrate and 10 mM EDTA at pH 6.5) and centrifuged at 2400 x *g* for 45 minutes. Platelets were resuspended in lysing buffer (0.15 M NaCl, 2 mM EDTA, 20 mM Tris, 2 mM N-Ethylmaleimide, 1% Triton X-100 and EDTA free protease inhibitors at pH 7.4) for 30 minutes at room temperature and stored at -80°C until further use.

Platelet lysates were thawed and centrifuged at 35 000 RPM for 2 hours using the Beckman Ultracentrifuge I8-80 (rotor Ti70), and solubilized platelet lysates were obtained. Wheat germ agglutinin (WGA) beads were incubated in the regeneration buffer (0.5 M NaCl, 1 mM Mg^{2+}, 1 mM Mn^{2+}, 1 mM Ca^{2+} and 1 mM Zn^{2+}) for 5 minutes at room temperature. The beads were centrifuged at 300 x *g* for 1 minute and washed with the wash buffer (0.15 mM NaCl, 2 mM EDTA, 20 mM Tris, 2 mM N-Ethylmaleimide, 0.1% Triton X-100 at pH 7.4). 10 mL of WGA beads were mixed with 100 mL of the solubilized platelet lysate for 2 hours at room temperature. The beads and the supernatant were added to a column and washed with the wash buffer. Proteins bound to the beads were eluted with the elution buffer (0.15 mM NaCl, 2 mM EDTA, 20 mM Tris, 2 mM N-Ethylmaleimide, 0.01% Triton X-100 and 2.5% N-acetyl-D-glucosamine at 7.4 pH). The elution fractions were collected, and absorbances were measured at 280 nm using the Eppendorf BioPhotometer.

The elution fractions were tested on SDS-PAGE under reducing conditions to analyze the protein contents.

3.4. Protein Concentration

Purified GPIIbIIIa and GPIbIX were concentrated using Amicon Millipore 3000 MWCO 50 mL tubes. The Amicon Millipore tube filter was prewet with 500 µl of Milli-Q-water and centrifuged at 3700 x *g* for 5 minutes. Protein samples were added to the tube and centrifuged at 3700 x *g* for 15 minutes. Concentrated protein samples were collected and stored at -20°C. The concentrations of proteins were further determined using a bicinchoninic assay (BCA) kit.

3.5. Peripheral Blood Mononuclear Cell Isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood samples from patients and healthy controls. Whole blood was centrifuged at 170 x *g* for 15 minutes, and PRP was harvested for autoantibody testing. PRP depleted blood was diluted with PBS at a 1:1 ratio and 25 mL of diluted blood were layered on 15 mL of Ficoll Histopaque 1077 followed by centrifugation at 400 x *g* for 30 minutes. PBMCs were harvested and washed once with PBS containing 1 unit/mL of heparin and was followed by another wash with AIM V serum-free media supplemented with 1% L-glutamine and 1% Penicillin-Streptomycin (supplemented AIM V media). PBMCs were centrifuged at 370 x *g* for 10 minutes between washes and resuspended in supplemented AIM V media.

3.6. Bone Marrow Mononuclear Cell Isolation

Bone marrow mononuclear cells (BMMCs) were isolated from bone marrow aspirate samples from patients and healthy controls. 10 mL of bone marrow aspirate samples (9 mL aspirate in 1 mL heparin/PBS) were filtered through a 70 µm filter to remove any bone fragments. The bone marrow aspirate was diluted at a 2:1 ratio with PBS containing 1 unit/mL of heparin. In two 15 mL tubes, 7.5 mL aliquots of the diluted bone marrow aspirate were layered on 4 mL of Ficoll Histopaque 1077 and centrifuged at 400 x *g* for 30 minutes. BMMCs were harvested and washed with PBS containing 1 unit/mL of heparin followed by another wash with supplemented AIM-V media. Cells were centrifuged at 370 x *g* for 10 minutes between washes and resuspended in supplemented AIM-V media.

3.7. Cell Counting

Cells were counted using TC20 Automated Cell Counter and Beckman Coulter ® Ac·T diff™ Analyzer. The cell count and the viability of the cells were determined using the Trypan blue dye in the TC20 Automated Cell Counter. Complete blood count (CBC) was also obtained from the Beckman Coulter® Ac·T diff™ Analyzer.

3.8. Mononuclear Cell Stimulation

PBMCs and BMMCs were stimulated to increase the number of ASCs. Mononuclear cells were stimulated with 2.5 µg/mL of Resiquimod (R848) and 10 ng/mL of IL-2 in supplemented AIM-V media. Mononuclear cells were also cultured in supplemented AIM-V media without the addition of stimulants as a negative control. For

the experiments involving IL-21 stimulation, mononuclear cells were stimulated with 2.5 µg/mL of R848, 10 ng/mL of IL-2 and 50 ng/mL of IL-21 in supplemented AIM-V media. The cells were cultured in 1 mL volume at a concentration of 1 x 10^6 mononuclear cells/mL in 24 well tissue culture plates for 6 days at 37°C with 5% CO_2. On day 6 or any other duration of stimulation (day 1 - day 10) as specified in the results section, the stimulated and unstimulated cell cultures were harvested, and the wells were rinsed with supplemented AIM-V media. The cells were centrifuged at 370 x *g* for 10 minutes, and the supernatants that the cells were cultured in were collected and stored at -80°C for autoantibody testing in the indirect ELISA. The cells were washed 2 times with supplemented AIM-V media.

3.9. Hybridoma Cell Culture

Raj-1 and TW-1 hybridoma cell lines were passaged 1:15 every three days in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, and 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The cells were cultured at 37°C with 5% CO_2. For the ELISPOT assay, the hybridoma cells were washed 2 times with supplemented RPMI and centrifuged at 370 x *g* for 10 minutes. Hybridoma cells were counted and cultured in the ELISPOT plate in supplemented RPMI media.

3.10. ELISPOT Assay

The ELISPOT assay was conducted to detect ASCs. Multiscreen filter polyvinylidene fluoride (PVDF) 96-well plates were briefly activated with 30µL of 70% ethanol for less than 1 minute and washed three times with distilled water (dH_2O).

Depending on the experiment, the wells were coated with 30 µg/mL of purified GPIIbIIIa, 30 µg/mL of purified GPIbIX, or 10 µg/mL of mouse anti-human IgG, IgM, and IgA antibodies in Tris-buffered saline (TBS) containing 0.5 mM $CaCl_2$ (TBS+Ca^{2+}) overnight at 4°C. The plates were washed five times with TBS+Ca^{2+} and blocked with TBS+Ca^{2+} containing 10% fetal bovine serum (FBS) for 2 hours at 37°C. The plates were washed 2 times with TBS+$Ca^{2+,}$ followed by the addition of mononuclear cells. 100 000 – 350 000 stimulated or unstimulated mononuclear cells were added in the antigen-coated wells and 10 000 – 100 000 mononuclear cells were added in anti-human IgG, IgM and IgA coated wells. Cells were incubated in the plates overnight at 37°C with 5% $CO_{2.}$ The following day, the cells were decanted, and the plates were incubated with TBS+Ca^{2+} containing 0.05% Tween 20 (TBS+Ca^{2+}+0.05% TW-20) for 10 minutes followed by 2 washes with TBS+Ca^{2+}+0.05% TW-20 and 4 washes with TBS+Ca^{2+}. Antibodies secreted by the cells were detected with 1 µg/mL of biotinylated mouse anti-human IgG, IgM or IgA antibodies in TBS+Ca^{2+} containing 2% FBS for 2 hours at 37°C. The plates were washed 4 times with TBS+Ca^{2+} followed by the addition of streptavidin conjugated to alkaline phosphatase for 1 hour at room temperature. Once the plates were washed, 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium (BCIP/NBT) substrate was added to the wells. The plates were protected from the light and incubated with the substrate until spots were visible in the wells (approximately 25 minutes). The front and the back of the plates were washed with distilled water and air dried. The plates were stored at room temperature and protected from light. The schematic diagram of the ELISPOT assay displays the steps involved in the detection of antibodies secreted by ASCs (Figure 2).

The following positive and negative controls were included on every ELISPOT assay conducted. Hybridoma cells were used as a positive control to check the integrity of purified GPIIbIIIa and GPIbIX and to ensure that the ELISPOT assay can detect antigen-specific ASCs. The ELISPOT plate wells were coated with 30 µg/mL of GPIIbIIIa, 30 µg/mL of GPIbIX and 10 µg/mL of goat anti-mouse IgG coating antibodies. 100 Raj-1 hybridoma cells and 100 TW-1 hybridoma cells were added to GPIIbIIIa, and GPIbIX coated wells, respectively and also to goat anti-mouse IgG coated wells. Secreted antibodies were detected with 1 µg/mL of biotinylated anti-mouse IgG antibodies. For the negative controls, cells were incubated in wells without coating antigens or antibodies, and AIM-V media was also incubated in wells coated with the antigens and the antibodies. Antigen-specific and total IgG tests were conducted in 4-7 replicate wells. Various factors of the ELISPOT assay protocol were altered and tested during the optimization of the assay as described in the results section.

3.10.1. ELISPOT Analysis

ELISPOT plates were analyzed and quantified using the Bio-Sys Bioreader 5000 and the CTL ImmunoSpot S6 Micro Analyzer. The reader scanned ELISPOT plates and spots were counted based on a combination of parameters set to characterize spots, including the size, diffusiveness, circularity, proximity between spots, sensitivity, background contrast, etc. Once the wells were counted, each well was manually checked to ensure that all of the visible spots were counted and that any artifacts (hair, debris, etc.) counted as spots were manually adjusted accordingly as a quality control step. Antigen-specific spots and total antibody spots were counted as a mean of all of the replicate wells.

Spots detected in the no coat wells (negative control) were subtracted from the number of spots detected in the experimental wells. Data were expressed as either the number of antigen-specific spots detected/100 000 mononuclear cells or as the % of antigen-specific spots relative to the number of total IgG ASCs detected.

3.11. GPIIbIIIa and GPIbIX Direct Antigen Capture Assay

The direct antigen capture assay was used to detect GPIIbIIIa and GPIbIX IgG autoantibodies bound to the antigens on platelets/megakaryocytes. PRP harvested from peripheral blood, and bone marrow aspirate from patients and healthy controls were centrifuged at 2000 x *g* for 10 minutes, and platelets were washed 2 times with ACD/PBS buffer. Platelets were counted and lysed with a minimum volume of 500 µl of lysing buffer (TBS/1% Triton X-100 + EDTA free protease inhibitors) at 300 000 platelets/µl. Lysate samples were stored at 4°C until the samples were used in the AC assay. Polystyrene 96-well plates were coated with 10 µg/mL of Raj-1 and TW-1 monoclonal antibodies overnight at 4°C. Plates were blocked with TBS + 2% BSA for 1 hour. Platelet lysates were added in duplicates to Raj-1, and TW-1 coated wells for 3 hours at room temperature. The plates were washed with TBS and incubated with anti-human IgG antibodies conjugated to alkaline phosphatase in blocking buffer for 1 hour at room temperature. The plates were washed with TBS + 0.05% TW-20 and TBS followed by the addition of the substrate containing 1 mg of p-nitrophenyl phosphate/1 mL of diethanolamine buffer. O.D. values were measured immediately at 405 nm every 1 minute for 60 minutes using the Bioteck Microplate Autoreader.

3.12. GPIIbIIIa and GPIbIX Indirect ELISA

Anti-GPIIbIIIa and anti-GPIbIX IgG autoantibodies were detected in the supernatants that the mononuclear cells were cultured in for a specific duration of stimulation. Polystyrene 96 well microtiter plates were coated with 10 µg/mL of affinity purified GPIIbIIIa and 10 µg/mL of affinity purified GPIbIX in bicarbonate buffer overnight at 4℃. The plates were blocked with blocking buffer (TBS + 2% BSA) for 2 hours at 37 ℃. Supernatants from BMMCs and PBMCs stimulated cultures were tested for autoantibodies from the following conditions: R848 and IL-2 stimulation, R848, IL-2, and IL-21 stimulation, and no stimulation. Supernatants were added to the wells in duplicates for 1.5 hours at 37℃. The plates were washed 3 times with TBS and antibodies bound to the antigens were detected with goat anti-human IgG antibodies conjugated to alkaline phosphatase in blocking buffer for 1 hour at room temperature. The plates were washed with wash buffer containing 0.05% TW-20 (TBS+0.05% TW20) followed by a TBS wash. The substrate (p-nitrophenyl phosphate in Diethanolamine buffer at pH 9.6) was added to the plate, and O.D. values were measured immediately at 405 nm every 1 minute for 60 minutes using the Bioteck Microplate Autoreader. Due to the lack of positive controls established for detecting antigen-specific antibodies in the supernatants using the indirect ELSA, serum from patients known to have anti-GPIIbIIIa and anti-GPIbIX antibodies were diluted 1/100 in AIM-V media and used in the assay. For the negative control, serum and supernatants from healthy controls and AIM-V media that the mononuclear cells were not cultured in were tested for the presence of anti-GPIIbIIIa and anti-GPIbIX autoantibodies.

3.13. B Cell Isolation

B cells were isolated from PBMCs by negative selection using the EasySep Human B cell Isolation Kit. 0.25 – 2 mL of 50 x 10^6 PBMCs/mL were added to a polystyrene round bottom tube and mixed with 50 µl of Cocktail Enhancer for every 1 mL of the initial cell sample volume. Similarly, 50 µl of the Isolation Cocktail was added for every 1 mL of the initial cell sample volume and mixed for 5 minutes at room temperature. 50 µl of RapidSpheres were added for every 1 mL of the initial cell sample volume followed by the addition of PBS + 2% FBS buffer to a final volume of 2.5 mL. The tube was placed on the EasySep magnet for 3 minutes at room temperature, and the cell suspension was poured off into another tube. The tube with the cell suspension was inserted into the magnet once more and incubated for 1 minute at room temperature. The enriched cell suspension was poured out and collected. Purified B cells were counted and used for flow cytometry to determine the purity of the B cells and were also used in the ELISPOT assay to quantify ASCs.

3.14. Immunophenotyping Isolated B Cells

To immunophenotype isolated B cells, 10^5 B cells were washed with staining buffer (PBS + 2% FBS) and stained with fluorescently labeled mouse anti-human IgG antibodies conjugated to specific fluorophores (APC CD45, PE CD19) in staining buffer for 30 minutes at 4°C. Stained cells were washed once with staining buffer and analyzed using the Beckman Coulter CytoFLEX Flow Cytometer. The experiments also included compensation controls, isotype controls and no colour controls for each set of samples. Flow cytometry data were analyzed using the FlowJo software. Compensation controls

were applied to take into account any spectral overlap between fluorophores used to stain the cells. The isotype controls were applied to ensure that the binding of the fluorescent antibodies is not due to the non-specific binding of antibodies.

3.15. Statistical Analysis

GraphPad Prism (Version 8.0) was used to produce graphs and perform statistical analysis on the data. Results are expressed as mean ± standard deviation (SD). A P value < 0.05 was determined to be statistically significant for all experiments. An unpaired t-test was used to compare differences between two distinct samples and a paired t-test was used to compare the differences of the same sample tested in two different conditions.

4.0. RESULTS:

4.1 Development and Optimization of the ELISPOT assay

4.1.1. Purifying Platelet GPIIbIIIa and GPIbIX

In order to develop an ELISPOT assay to detect autoreactive ASCs that secretes platelet-specific autoantibodies, GPIIbIIIa and GPIbIX were purified from freshly outdated human platelets. The protein contents of the eluted fractions were assessed using SDS-PAGE under reduced conditions (Figure 3). Distinct bands of proteins were present at 110 kDa and 125 kDa that corresponds to the molecular weight of GPIIb and GPIIIa, respectively (Figure 3a). Elution fractions from GPIbIX purification contained protein bands that correspond to GPIbα at 135 kDa, GPIbβ at 26 kDa, and GPIX at 20 kDa (Figure 3b).

4.1.2. Validating GPIIbIIIa and GPIbIX Specific ELISPOT Assay Using Hybridoma Cells

One of the challenges of optimizing an ELISPOT assay to detect anti-GPIIbIIIa and anti-GPIbIX ASCs was the lack of known human ASCs that secrete antibodies to the platelet antigens. To optimize the ELISPOT assay, we used Raj-1 and TW-1 hybridoma cell lines that secrete mouse monoclonal IgG antibodies to an epitope formed by GPIIbIIIa complex, and GPIbα, respectively[54]. First, the concentration of the antigens required to detect ASCs was determined by coating the ELISPOT plate wells with 0 µg/mL - 100 µg/mL of GPIIbIIIa and GPIbIX and incubating approximately 100 Raj-1 and 100 TW-1 cells overnight, respectively (Figure 4a). Spots were detected in all of the wells except for

the no coat wells (0 µg/mL). The use of lower concentrations of the antigens resulted in the formation of larger, and more diffuse spots as some spots overlapped. Whereas, using higher concentrations of the antigens resulted in smaller and less diffuse (tight) spots. Approximately 30 µg/mL of GPIIbIIIa and 30 µg/mL of GPIbIX was determined to be the optimal concentration of the antigens required to minimize formation of large, overlapping spots while also ensuring that the spots formed by ASCs that secrete low amounts of antibodies are still detectable. This is to ensure that the ELISPOT plate reader detects human ASCs that secrete varying amounts of antibodies and form spots with varying characteristics (e.g., spot size).

To assess the sensitivity of the assay to detect antigen-specific ASCs, hybridoma cells were incubated in the ELISPOT plate wells coated with the antigens and in the positive control wells coated with anti-mouse IgG antibodies overnight. 98.7% of Raj-1 cells were detected in the GPIIbIIIa specific ELISPOT assay since 73 ± 11 spots were detected in GPIIbIIIa coated wells and 74 ± 10 spots were detected in anti-mouse IgG coated wells (Figure 4b). Similarly, 97.1% of TW-1 cells were detected in GPIbIX specific ELISPOT assay since 91 ± 10 spots were detected in GPIbIX coated wells and 95 ± 10 spots were detected in anti-mouse IgG coated wells. Spots were not detected when the hybridoma cells were incubated in the negative control wells that did not contain coating antibodies or antigens (no coat). These findings indicate that the antigen-specific ELISPOT assay is able to detect hybridoma cells with high sensitivity and that the spots detected in the antigen coated wells are due to specific binding of antibodies secreted by the cells to the coating antigens. In contrast, the low sensitivity of the assay was observed when Raj-1 cells were

incubated overnight in wells coated with partially degraded GPIIbIIIa. Only 6.9% of Raj-1 cells were detected since 5 ± 4 spots and 72 ± 2 spots were detected in the partially degraded GPIIbIIIa, and anti-mouse IgG coated wells, respectively (Figure 4c). Majority of the spots were too diffuse and indistinguishable for the ELISPOT plate reader to detect as distinct spots. Reducing the Raj-1 cell incubation time to 4 hours increased the number of spots detected, however only 53.8% of Raj-1 cells were detected in the GPIIbIIIa coated wells. Thus, to assess the integrity of the coating antigens and the sensitivity of the assay to detect ASCs, hybridoma cells were detected in every ELISPOT assay conducted as a positive technical control.

4.1.3. Interference of Antigen-specific Spot Formation by Platelets and PBMCs

In previous studies, autoreactive ASCs were detected in ITP patients by incubating unstimulated PBMCs in the ELISPOT plate wells for 4 hours[66,68]. When we incubated unstimulated PBMCs in wells coated with anti-human IgG antibodies and antigen-coated wells, total IgG ASCs were rarely detected, let alone antigen-specific ASCs. Since some patients had detectable total IgG ASCs but did not have antigen-specific ASCs, we tested whether the presence of platelets and/or PBMCs could potentially interfere with the formation of antigen-specific spots. To determine whether platelets absorb the antibodies secreted by Raj-1 and TW-1 hybridoma cells, 400 000 platelets were incubated with 100 hybridoma cells in GPIIbIIIa, and GPIbIX coated wells, respectively. The hybridoma cells were also incubated with 400 000 PBMCs in the antigen-coated wells. The presence of platelets or PBMCs did not reduce the number of spots formed by hybridoma cells (Figure

5). This suggests that the lack of antigen-specific spots produced by PBMCs from ITP patients is not due to platelets or PBMCs interfering with the formation of spots.

4.2 Enhancing IgG ASCs in PBMCs

4.2.1. Stimulating PBMCs with R848 and IL-2 to Enhance Total IgG ASCs

In order to optimize the ELISPOT assay to detect anti-GPIIbIIIa and anti-GPIbIX IgG ASCs, we stimulated PBMCs with a polyclonal inducer, R848, and IL-2 to enhance the proportion of total IgG ASCs. The stimulants have been used previously to enhance detection of total IgG ASCs by stimulating PBMCs for 3 days or 6 days[84]. Thus, we incubated PBMCs in the ELISPOT plate wells coated with anti-human IgG antibodies on day 0 (unstimulated PBMCs), day 3 of stimulation and day 6 of stimulation for 4 hours and overnight (Figure 6a). The highest number of total IgG ASCs were detected on day 6 of stimulation since 158 ± 5 spots and 174 ± 1 spots were detected when PBMCs were incubated in anti-human IgG coated wells for 4 hours and overnight, respectively. Whereas, spots were not detected on day 0, and only 23 ± 5 spots and 39 ± 9 spots were detected on day 3 of stimulation when PBMCs were incubated for 4 hours and overnight, respectively. Additionally, overnight incubation of stimulated PBMCs resulted in more spots than a 4-hour incubation. We also assessed the size of the spots formed by ASCs to maximize the number of spots detected by the ELISPOT plate reader (Figure 6b). Overnight incubation of PBMCs on day 3 of stimulation produced 16.5 ± 2.6 x 10^{-3} mm^2 spots, and on day 6 of stimulation produced 17.3 ± 1.4 x10^{-3} mm^2. Whereas, smaller spots were produced when PBMCs were incubated for 4 hours on both day 3 and day 6 of stimulation since the spots were only 5.4 ± 2.6 x 10^{-3} mm^2 and 9.7 ± 3.6 x 10^{-3} mm^2, respectively. Findings from these

experiments indicate that stimulating PBMCs for 6 days with R848 and IL-2 and incubating the cells on the ELISPOT plate wells overnight will maximize the number and increase the size of spots formed by ASCs.

4.2.2. Time Course Detecting Anti-GPIIbIIIa, Anti-GPIbIX, and Total IgG ASCs After PBMCs Stimulation

Time course experiments involving the duration of PBMCs stimulation were conducted to determine if the number of total IgG ASCs continues to increase after day 6 of stimulation. PBMCs were tested for total IgG ASCs and antigen-specific ASCs daily from before stimulation until day 7 (n=2) or day 10 (n=2) of stimulation. For 3 of the samples, the highest number of total IgG spots were detected on day 6 of stimulation (1245 ± 223 total IgG ASCs/10^5 PBMCs, 525 ± 105 total IgG ASCs/10^5 PBMCs, and 765 ± 204 total IgG ASCs/10^5 PBMCs). Whereas, 1 of the samples produced the highest number of total IgG ASCs on day 7 of stimulation (738 ± 192 total IgG ASCs/10^5 PBMCs) (Figure 7a).

PBMCs samples were also tested for the presence of antigen-specific ASCs during the time course experiment as a proof of principle to determine if platelet-specific ASCs are detectable in patients with a high titer of platelet-specific antibodies in the serum. We tested PBMCs from a patient with GT known to have a high titer of anti-GPIIbIIIa IgG alloantibodies in circulation (O.D. > 2.00 in the indirect antigen capture assay). Anti-GPIIbIIIa IgG spots were detected only after day 4 of stimulation and were the most abundant on day 8 of stimulation at a frequency of 14.0 ± 1.9 anti-GPIIbIIIa IgG ASCs/10^5

PBMCs (2.77 ± 0.38% of the total IgG ASCs) (Figure 7b and c). The visual representation of the wells display spots formed on day 0 to day 10 of stimulation when 300 000 PBMCs were incubated in GPIIbIIIa coated wells, and 10 000 PBMCs were incubated in anti-human IgG coated wells (Figure 7f). Whereas, the GT patient had less than 0.2 ± 0.3 anti-GPIbIX IgG ASCs/10^5 PBMCs (< 0.1% of the total IgG ASCs) (Figure 7d and e). Next, we tested a patient with BSS who did not have platelet-specific antibodies, and during the time course, less than 0.5 ± 0.7 anti-GPIIbIIIa IgG ASCs/10^5 PBMCs (0.08 ± 0.1 % of the total IgG ASCs) were detected, and anti-GPIbIX ASCs were not detected. We also tested PBMCs from 2 healthy controls, and both samples did not produce antigen-specific spots except on day 7 of stimulation when one of the healthy donors produced 0.3 ± 0.6 anti-GPIbIX IgG ASCs/10^5 PBMCs (0.07 ± 0.1 % of the total IgG ASCs). Additionally, the GT patient's PBMCs were also added to the wells coated with 0 µg/mL - 50 µg/mL of GPIIbIIIa on day 6 of stimulation (Figure 7g). Although anti-GPIIbIIIa ASCs were detected in all of the wells, the size of spots formed decreased from 70.8 ± 25.4 x10^{-3} mm^2 to 18.7 ± 3.5 x10^{-3} mm^2 when wells were coated with 30 µg/mL and of 40µg/mL of GPIIbIIIa, respectively.

As stimulated cells were used for the ELISPOT assay, the supernatants that the PBMCs were cultured in during stimulation were tested for the presence of antigen-specific IgG antibodies in the indirect AC ELISA. The supernatants from GT patient's PBMCs had anti-GPIIbIIIa IgG antibodies at an O.D. of 0.45 ± 0.05 on day 1 of stimulation and increased to the highest O.D. of 1.40 ± 0.16 on day 8 of stimulation (Figure 8a). Whereas, the healthy control had the highest O.D. of 0.17 ± 0.02. The supernatants from the BSS patient who did not have detectable anti-GPIbIX ASCs had the highest O.D. of 0.12 ± 0.00,

which is similar to the highest O.D. of 0.10 ± 0.00 detected in the supernatants of the healthy control (Figure 8b). These time course experiments confirmed that day 6 of PBMCs stimulation produced the highest number of total IgG ASCs in most samples. Additionally, as a proof of principle, we showed that the GT patient with a high titer of anti-GPIIbIIIa antibodies in the serum had a high frequency of anti-GPIIbIIIa IgG ASCs as detected using the ELISPOT assay. This was further confirmed by the presence of high concentrations of anti-GPIIbIIIa IgG antibodies in the cell culture supernatants.

4.2.3. Non-specific Binding of IgM Antibodies in the ELISPOT Assay

The majority of autoantibodies detected in ITP patients are of IgG class, but IgM autoantibodies have also been detected in a subset of patients[62,63]. As we tested PBMCs for antigen-specific IgG ASCs in ITP patients and healthy donors, we also tested PBMCs for the presence of antigen-specific IgM ASCs. Unlike detection of IgG spots, IgM spots were detected in the antigen-coated wells and the no coat negative control wells. For example, we detected 95 ± 6 GPIIbIIIa IgM ASCs/10^5 PBMCs, 58 ± 7 GPIbIX IgM ASCs/10^5, and 2190 ± 151 total IgM ASCs/10^5 PBMCs (Figure 9a). However, 1060 ± 208 IgM ASCs/10^5 PBMCs were also detected in the no coat wells, indicating that the spots detected in the antigen-coated wells lack specificity. In contrast, we detected 180 ± 45 total IgG ASCs/10^5 PBMCs, but spots were not detected in antigen-coated wells or the negative control wells. In another example, IgM spots were detected in wells coated with IgG and IgA coating antibodies and in the no coat wells (Figure 9b). Whereas, IgA and IgG spots were only detected in the wells coated with anti-human IgA and anti-human IgG antibodies, respectively. Spots were not detected in wells that were not incubated with cells regardless

of the coating antibodies or the detection antibodies used. This experiment confirms that IgM antibodies secreted by IgM ASCs bind non-specifically in the ELISPOT assay.

We attempted to eliminate the non-specific binding of IgM spots in the no coat negative control wells by testing various factors involved in the assay. These factors were tested using PBMCs from multiple donors. Since the non-specific binding of IgM spots varies between donors, the results are displayed as a visual representation of the wells for qualitative analysis of the presence or absence of IgM spots detected in the total IgM and the no coat wells. First, we attempted to reduce the PVDF membrane binding capacity of the wells by not activating the membrane with 70% ethanol and using nitrocellulose membrane plates known to have a lower binding capacity. Reducing the binding capacity of the membrane did not eliminate the non-specific binding of IgM antibodies on the no coat wells (Figure 10a). Reducing the incubation time of PBMCs in the wells for 2 hours and 4 hours did not prevent non-specific binding of IgM antibodies (Figure 10b). The shorter incubation time resulted in smaller and fewer IgM spots in both the no coat wells and total IgM wells. Additionally, IgM ASCs produce spots with a wide range of sizes, so a size cut off could not be used to differentiate between the formation of non-specific IgM spots and specific IgM spots. We also tested multiple blocking agents such as FBS, BSA, higher grade BSA, goat serum, and superblock (western blot blocking agent) as well as no blocking. Non-specific IgM spots were observed in the no coat wells regardless of the type of blocking buffer used (Figure 10c). In addition to these factors, incorporating stringent wash steps with high salt concentrations or TW-20 detergent washes did not reduce the non-specific binding of IgM spots (data not shown). Non-specific binding of IgM spots

were also observed in stimulated and unstimulated PBMCs. Since non-specific binding of IgM spots were not eliminated, anti-GPIIbIIIa and anti-GPIbIX IgM autoreactive ASCs were not assessed in ITP patients.

4.3. Detecting Autoantibodies and Autoreactive B Cells in the Periphery of ITP Patients and Healthy Donors

The number of total IgG ASCs were detected in unstimulated and 6 day stimulated PBMCs (Figure 11a) (n=33). 7.46 ± 14.71 total IgG ASCs/10^5 PBMCs detected in unstimulated PBMCs (day 0) was significantly lower than 651.00 ± 1260.00 total IgG ASCs/10^5 PBMCs detected in stimulated PBMCs (p=0.006). Visual representation of the wells displays an increased number of spots on day 6 of stimulation compared to day 0 (Figure 11b).

We also tested 23 ITP patients and 13 healthy donors for the presence of anti-GPIIbIIIa and anti-GPIbIX IgG ASCs in stimulated PBMCs (Figure 12 a and b). Anti-GPIIbIIIa IgG ASC(s) were detected in 4/23 ITP patients at a frequency of 0.04 ± 0.11 anti-GPIIbIIIa IgG ASCs/10^5 PBMCs (0.04 ± 0.15 % of total IgG ASCs) and was not significantly different than 0.12 ± 0.22 anti-GPIIbIIIa IgG ASCs/10^5 PBMCs (0.02 ± 0.03% of total IgG ASCs) detected in 5/13 healthy donors (p=0.127). Whereas, 3/23 ITP patients had anti-GPIbIX IgG ASCs at a mean frequency of 0.01 ± 0.04 anti-GPIbIX IgG ASCs/10^5 PBMCs (0.01 ± 0.04% of total IgG ASCs) and was not significantly different than the mean frequency of 0.01 ± 0.02 anti-GPIbIX IgG ASCs/ 10^5 PBMCs (0.0002 ± 0.001% of total IgG ASCs) detected in 1/13 healthy donors (p=0.461). 15 of these ITP patients were also

tested for anti-GPIIbIIIa and anti-GPIbIX platelet-bound IgG autoantibodies. Platelets were harvested from the peripheral blood samples and solubilized to test for the presence of platelet-bound autoantibodies using the direct AC assay. The pre-established cut-off value was determined as 2SD above the mean O.D. of healthy controls. After applying the cut-off O.D. of 0.21, 6/15 (40%) patients had anti-GPIIbIIIa IgG autoantibodies, and 5/15 (33%) patients had anti-GPIbIX IgG autoantibodies (Figure 12c). All of the patients with anti-GPIbIX autoantibodies also had anti-GPIIbIIIa autoantibodies.

4.4. Enhancing Antigen-specific ASCs in PBMCs

4.4.1. Increasing the Number of B Cells Tested in the ELISPOT Assay

To enhance detection of anti-GPIIbIIIa and anti-GPIbIX ASCs, we increased the number of B cells tested in the ELISPOT assay. First, we increased the concentration of PBMCs added to the wells from 100 000 PBMCs/well to 350 000 PBMCs/well and also increased the number of replicates to maximize detection of antigen-specific ASCs. We also isolated B cells (including plasma cells) from PBMCs to increase the concentration of ASCs in each well. In the first experiment, 11.9% of PBMCs were recovered after purifying B cells from PBMCs (Table 1). Flow analysis showed that the purity of the B cells was 99.9% (1131 CD45+ CD19+ cells/ 1132 CD45+ cells) (Figure 13a). After the purified B cells were stimulated for 6 days, only 13.0% of the cells were CD19+ B cells (629 CD45+ CD19+ cells/ 4839 CD45+ cells) (Figure 13b). Stimulation of B cells also increased the number of CD45+ cells by 4.2 fold. In the ELISPOT assay, 14 ± 2 total IgG ASC/10^4 B cells were detected in the B cells that were initially isolated and then stimulated, and $25 \pm$

6 total IgG ASCs/10^4 PBMCs were detected in stimulated PBMCs (Table 1). In another experiment, 2.2% of PBMCs were recovered after purifying B cells. 2 ± 0 total IgG ASCs/10^4 B cells were detected after the isolated B cells were stimulated, 30 ± 3 total IgG ASCs/10^4 B cells were detected from the B cells that were isolated from stimulated PBMCs and 17 ± 2 total IgG ASCs/10^4 PBMCs were detected in stimulated PBMCs. Findings from these experiments showed that isolating B cells from stimulated PBMCs results in a higher number of IgG ASCs rather than purifying B cells and then stimulating them.

4.4.2. Stimulating PBMCs with R848, IL-2, and IL-21

We also stimulated mononuclear cells with IL-21 in combination with R848 and IL-2 to enhance the frequency of antigen-specific IgG ASCs. IL-21 is a cytokine secreted by activated T cells and is known to be involved in the production of autoantibodies in autoimmune diseases[85,86]. Additionally, elevated levels of IL-21 has been detected in ITP patients[87]. PBMCs from ITP patients (n=7) and healthy controls (n=7) were tested in the ELISPOT assay before stimulation, after day 6 stimulation with R848 and IL-2, and after 6 day stimulation with R848, IL-2, and IL-21. The number of total IgG ASCs detected after stimulation with R848, and IL-2 was significantly higher than IgG ASCs detected in unstimulated PBMCs (p=0.028). IgG ASCs detected in PBMCs stimulated with R848, IL-2 and IL-21 were significantly higher than the total IgG ASCs detected in PBMCs stimulated with R848 and IL-2 (p=0.004) and also significantly higher than unstimulated PBMCs, (p=0.005) (Figure 14a). The samples stimulated with R848, IL-2 and IL-21 were also tested for the presence of antigen-specific IgG ASCs. 1/7 ITP patients had 1.2 ± 1.7 anti-GPIIbIIIa IgG ASCs/10^5 PBMCs (0.02% of total IgG ASCs) and in 1/7 healthy donors

had 0.1 ± 0.2 anti-GPIIbIIIa IgG ASCs/10^5 PBMCs (0.05% of total IgG ASCs) (Figure 14b). None of the ITP patients or healthy donors had detectable anti-GPIbIX ASCs. Although PBMCs stimulation with R848, IL-2, and IL-21 significantly increased the number of total IgG ASCs, detection of anti-GPIIbIIIa and anti-GPIbIX ASCs was not enhanced.

4.4.3. Comparison of Anti-GPIIbIIIa and Anti-GPIbIX IgG Autoantibodies in the Supernatants of Stimulated Mononuclear Cells

Next, antigen-specific IgG autoantibodies were detected in the media supernatants that the ITP patient's PBMCs were cultured in during the stimulation period. PBMCs were stimulated with R848 and IL-2 for 6 days and to increase the concentration of autoantibodies secreted by autoreactive cells in the cell culture supernatants, PBMCs were also stimulated for 10 days (n=5). There was no significant difference in the presence of anti-GPIIbIIIa, and anti-GPIbIX IgG autoantibodies in the supernatants of PBMCs stimulated for 6 days and 10 days (GPIIbIIIa p=0.806, GPIbIX p=0.236) (Figure 15a and b). To determine whether the addition of IL-21 enhances the production of autoantibodies, we compared the presence of antigen-specific IgG autoantibodies in the supernatants of PBMCs stimulated with R848 and IL-2 to the supernatants of PBMCs stimulated with R848, IL-2, and IL-21 for 6 days (n=5). There was a significant increase in the presence of anti-GPIIbIIIa autoantibodies when PBMCs were stimulated with R848, IL-2, and IL-21 (p=0.019) (Figure 15c). However, there was no significant difference in the presence of anti-GPIbIX autoantibodies (p=0.850) (Figure 15d).

4.5. Detecting Autoantibodies and Autoreactive B Cells in the Bone Marrow and the Peripheral Blood Compartments

4.5.1. Anti-GPIIbIIIa and Anti-GPIbIX IgG Autoreactive ASCs in the Bone Marrow and the Peripheral Blood

We tested 12 ITP patients, 3 non-immune thrombocytopenic control patients, and 5 healthy donors for the presence of anti-GPIIbIIIa and anti-GPIbIX autoantibodies and autoreactive ASCs in the bone marrow and the peripheral blood compartments. Initially, we also tested 7 sets of bone marrow aspirate and the peripheral blood samples which were not included in the results as the ELISPOT assay was being optimized and partially degraded GPIIbIIIa was used. Mononuclear cells (PBMCs and BMMCs) were isolated, and GPIIbIIIa, GPIbIX, and total IgG ASCs were detected before stimulation and on day 6 of stimulation. In addition to total IgG ASCs, we also detected total IgA and total IgM ASCs in the bone marrow of 19 samples (Figure 16). 127.5 ± 181.4 total IgG ASCs/10^5 PBMCs detected before stimulation of BMMCs was significantly lower than 468.8 ± 627.3 total IgG ASCs/10^5 PBMCs detected on day 6 of stimulation ($p=0.027$). Similarly, 771.5 ± 834.4 total IgM ASCs/10^5 PBMCs detected before stimulation was significantly lower than 3085.0 ± 2298.0 total IgM ASCs/10^5 PBMCs detected after stimulation ($p=0.0003$). Whereas, there was no significant increase in 204.3 ± 247.8 total IgA ASCs/10^5 PBMCs detected before stimulation to 294.7 ± 354.7 total IgA ASCs/10^5 PBMCs detected after stimulation ($p=0.324$). Representative wells display total IgG, IgA and IgM spots formed by ASCs in the bone marrow before and after stimulation (Figure 16d).

Next, we compared the frequency of anti-GPIIbIIIa and anti-GPIbIX ASCs using unstimulated and stimulated BMMCs. The frequency of antigen-specific spots is expressed as the mean ± SD of a number of spots/10^5 mononuclear cells. In the unstimulated BMMCs, anti-GPIIbIIIa ASCs were detected in the bone marrow of 3/12 ITP patients (0.19 ± 0.49 anti-GPIIbIIIa ASCs/10^5 BMMCs) and 1/5 healthy controls (0.17 ± 0.38 anti-GPIIbIIIa ASCs/10^5 BMMCs) (Figure 17a). Anti-GPIbIX ASCs were detected in the bone marrow of only 2/12 ITP patients (0.06 ± 0.16 anti-GPIbIX ASCs/10^5 BMMCs) (Figure 17b). After stimulation of BMMCs, 2/12 ITP patients had anti-GPIIbIIIa ASCs (0.01 ± 0.03 anti-GPIIbIIIa ASCs/10^5 BMMCs), 1/3 non-immune thrombocytopenic patient had anti-GPIIbIIIa ASCs (0.43 ± 0.75 anti-GPIIbIIIa ASCs/10^5 BMMCs) and 1/3 non-immune thrombocytopenic patient had anti-GPIbIX ASCs (0.33 ± 0.58 anti-GPIbIX ASCs/10^5 BMMCs). When we tested unstimulated PBMCs, anti-GPIIbIIIa ASCs were detected in 4/12 ITP patients (0.07 ± 0.12 anti-GPIIbIIIa ASCs/10^5 PBMCs), 1/3 non-immune thrombocytopenic patients (0.07 ± 0.13 anti-GPIIbIIIa ASCs/10^5 PBMCs), and 1/5 healthy controls (0.01 ± 0.02 anti-GPIIbIIIa ASCs/10^5 PBMCs). Anti-GPIbIX ASCs were detected in only 2/12 ITP patients (0.09 ± 0.29 anti-GPIbIX ASCs/10^5 PBMCs) in unstimulated PBMCs. Antigen-specific ASCs were not detected in stimulated PBMCs from patients or controls.

4.5.2. Anti-GPIIbIIIa and Anti-GPIbIX IgG Autoantibodies in the Bone Marrow and the Peripheral Blood

The direct antigen capture assay was used to detect anti-GPIIbIIIa and anti-GPIbIX IgG autoantibodies bound to platelets in the peripheral blood and platelets and/or megakaryocytes in the bone marrow. The pre-established cut-off O.D. of 0.21 (2SD above the mean O.D. of healthy controls) was applied, and 4/12 ITP patients were positive for anti-GPIIbIIIa IgG autoantibodies in the bone marrow whereas 3/12 were positive in the peripheral blood (Figure 18a). 3/12 ITP patients were positive for anti-GPIbIX IgG autoantibodies in the bone marrow, and 5/12 were positive in the peripheral blood (Figure 18b). 3/12 ITP patients had detectable autoantibodies in both compartments, and 5/12 ITP patients did not have detectable autoantibodies in either of the compartments (Table 5). 2/12 ITP patients had autoantibodies only in the bone marrow, and 1/12 ITP patients had autoantibodies only in the peripheral blood. 5/12 patients had GPIIbIIIa and/or GPIbIX autoantibodies in the bone marrow, and 5/12 patients had autoantibodies in the peripheral blood, resulting in a sensitivity of 42% in each compartment. Examining both compartments increased the sensitivity to 58% since 7/12 ITP patients had detectable autoantibodies in the bone marrow and/or the peripheral blood. Anti-GPIIbIIIa and anti-GPIbIX autoantibodies were not detected in the bone marrow or the peripheral blood of non-immune thrombocytopenic controls and healthy controls (Figure 18, Table 3 and 4).

4.5.3. Anti-GPIIbIIIa and Anti-GPIbIX IgG Autoantibodies in the Supernatants from Mononuclear Cells

When BMMCs and PBMCs were stimulated for 6 days and used in the ELISPOT assay, the supernatants that the cells were cultured in were harvested and tested for the presence of antigen-specific IgG autoantibodies using the indirect ELISA. ELISA plates were coated with purified GPIIbIIIa, and GPIbIX, incubated with supernatants and autoantibodies bound to the antigens were detected. The cut-off values were established as 2SD above the mean O.D. of healthy controls for each antigen in the bone marrow and the peripheral blood compartments. The cut-off for GPIIbIIIa IgG antibodies in the BMMCs supernatants was O.D. of 0.31, and 2/12 ITP patients, 1/3 non-immune thrombocytopenic controls and 0/5 healthy controls were positive (Figure 19a). The cut-off for GPIbIX IgG autoantibodies in the BMMCs supernatants was O.D. of 0.24, and 3/12 ITP patients, 0/3 non-immune thrombocytopenic controls and 0/5 healthy controls were positive (Figure 19b). For the PBMCs, the cut-off for GPIIbIIIa IgG autoantibodies was an O.D. of 0.46, and 1/12 ITP patients, 0/3 non-immune thrombocytopenic controls and 0/5 healthy controls were positive (Figure 19c). Whereas, the cut-off for GPIbIX IgG autoantibodies in the PBMCs supernatants was O.D. of 0.29 and 1/12 ITP patients, 0/3 non-immune thrombocytopenic controls and 0/5 healthy controls were positive (Figure 19d).

5. DISCUSSION:

Autoantibodies are a hallmark of ITP and are known to mediate platelet destruction and result in thrombocytopenia[26]. Autoantibodies are detected in the peripheral blood of only 40-50% of ITP patients, suggesting that either the assays used to detect autoantibodies have a low sensitivity, or the patients truly lack autoantibodies. This further suggests that mechanisms aside from autoantibody-mediated platelet destruction are involved in the pathophysiology of ITP and one of which may include platelet underproduction. Low platelet turnover has been observed in ITP patients, and several studies have suggested that autoantibodies might be sequestered in the bone marrow where autoantibodies may impair platelet production[22,37,38,40,43,88,89]. Thus, one of our aims was to compare the presence of autoantibodies in the bone marrow and the peripheral blood compartments of ITP patients. Another aim was to compare the presence of autoreactive ASCs in both of the compartments since detecting autoreactive ASCs has shown to be diagnostically more sensitive than detecting autoantibodies[66,67,69]. Thus, we also optimized a GPIIbIIIa and GPIbIX specific ELISPOT assay and attempted to enhance detection of autoreactive ASCs in ITP patients.

5.1. Optimizing Antigen-specific ELISPOT Assay and Enhancing Detection of Autoreactive B Cells

We optimized and validated the ELISPOT assay to detect autoreactive ASCs in ITP patients using purified GPIIbIIIa and GPIbIX along with Raj-1 and TW-1 hybridoma cells, respectively. The antigen-specific ELISPOT assay detected spots formed by the hybridoma

cells with high sensitivity and specificity, indicating the potential of the assay to detect human antigen-specific ASCs. The antibodies produced by these hybridoma cells have also been recommended to be implemented in the platelet autoantibody testing assays (antigen capture assays) by the International Society on Thrombosis and Haemostasis (ISTH) due to the high affinity and high specificity of the antibodies to the respective platelet antigens[54]. Many other studies have also used hybridoma cell lines as a tool to develop highly sensitive B cell ELISPOT assays to detect human ASCs of interest[90,91].

When we tested unstimulated PBMCs from ITP patients, anti-GPIIbIIIa and anti-GPIbIX specific spots were not detected, indicating that autoreactive plasma cells were not present in the peripheral blood of ITP patients. Whereas in a previous study, 5.4 ± 5.7 GPIIbIIIa specific ASCs/10^5 PBMCs and 3.0 ± 3.3 GPIb specific ASCs/10^5 PBMCs were detected when unstimulated PBMCs were tested from 114 ITP patients[66]. Similarly, in another study, 6.5 ± 4.1 GPIIbIIIa specific ASCs/10^5 PBMCs were detected when unstimulated PBMCs were tested from 63 ITP patients[67]. The findings from these studies indicate that autoreactive plasma cells were present and that autoantibodies were actively being secreted in the peripheral blood of most ITP patients. Furthermore, in our study, the lack of plasma cells detected in unstimulated PBMCs is likely because the ASCs are present at low frequencies in the peripheral blood. The majority of the B cells in the peripheral blood consists of naïve B cells and memory B cells, both of which do not secrete antibodies[9]. Whereas, the antibody-secreting cells are plasma cells/plasmablasts and consists of less than 2% of the B cells[28]. Since plasma cells only survive for a short duration in circulation, the presence of autoreactive plasma cells in unstimulated PBMCs reflect in

vivo secretion of autoantibodies, which may indicate the activity of the disease. A study comparing various combinations of PBMC stimulants also showed that IgG ASCs were not detectable in unstimulated PBMCs[84]. In another study, the majority of autoreactive B cells in the peripheral blood of ITP patients were suggested to be memory B cells rather than plasma cells[30]. This is because depletion of CD19+ cells in the PBMCs reduced production of anti-GPIIbIIIa autoantibodies and depletion of CD19+ cells in the spleen did not impact autoantibody production. Since memory B cells are CD19+ and plasma cells are not, the study suggested that the autoreactive B cells in the peripheral blood of ITP patients are mostly autoreactive memory B cells and that the autoreactive plasma cells tend to reside in the spleen.

Next, we enhanced the proportion of total IgG ASCs by stimulating PBMCs with R848 and IL-2, which was previously shown to enhance the proportion of IgG ASCs effectively[84]. R848 is a polyclonal inducer that activates T cells by binding to the toll-like receptor (TLR) 7 and TLR 8 which in combination with IL-2 induces memory B cells to proliferate and differentiate into plasma cells[92]. Since memory B cells have specificity to an antigen and are the second most abundant subtype of B cells in the peripheral blood (31% of B cells), we stimulated PBMCs to promote differentiation of memory B cells into plasma cells so the autoreactive memory B cells can be detected in the ELISPOT assay[42]. Stimulation of PBMCs significantly increased the proportion of total IgG ASCs, however, anti-GPIIbIIIa and anti-GPIbIX ASCs were detected at low frequencies and that too in a small subset of the ITP patients. In contrast to the previous studies, a small proportion of ITP patients were observed to have a low frequency/undetectable autoreactive plasma cells

especially with specificity to GPIb[56,66]. Autoreactive ASCs have also been detected in other autoimmune diseases. Patients with severe conditions of pemphigus vulgaris had detectable anti-desmoglein 3 (Dsg3) ASCs in unstimulated PBMCs at a frequency of 1.3-2.3 anti-Dsg3 ASCs/10^5 PBMCs and were not detected in patients with mild conditions of the disease[91]. In SLE patients, the presence of anti-dsDNA ASCs was associated with high disease activity[93]. Antigen-specific ASCs were also present at low frequencies in patients with hemophilia A where anti-FVIII ASCs were present at 0.02-0.28% of total IgG ASCs in stimulated PBMCs[24]. Similarly, an anthrax vaccine was shown to activate protective antigen-specific memory B cells, which were present at 0.05%-2.00% of total IgG ASCs in stimulated PBMCs[25]. In another study, 12 healthy individuals who have had prior exposure to tetanus toxoid were examined for tetanus-specific memory B cells, and only 50% (6/12) of the individuals had detectable memory B cells ranging from 0.03-0.37% of total IgG ASCs. In addition to the low frequencies of autoreactive ASCs detected in the peripheral blood of ITP patients in our study, these examples also indicate that antigen-specific ASCs are present at low frequencies in other diseases or vaccine-induced responses.

Anti-GPIIbIIIa and anti-GPIbIX antibodies are not only produced as an autoimmune response but can also be induced by platelet transfusions in some individuals[10]. For example, 25% of the GT patients who received platelet transfusions are known to produce anti-GPIIbIIIa alloantibodies. This is because the GT patient's platelets lack GPIIbIIIa or have GPIIbIIIa with qualitative defects. Thus, when the patients are exposed to platelets with normal GPIIbIIIa during platelet transfusions, an allogeneic

immune response might be induced, resulting in the production of allogeneic plasma cells and memory B cells. When we tested PBMCs from a GT patient, anti-GPIIbIIIa ASCs were detected only after PBMCs were stimulated, indicating that the patient did not have anti-GPIIbIIIa plasma cells in vivo and that memory B cells were present. This indicates that the patient had an allogeneic response to GPIIbIIIa likely when the patient was immunized during a platelet transfusion several years ago, and even though anti-GPIIbIIIa plasma cells only survive for a short duration, anti-GPIIbIIIa memory B cells were still present in circulation. Another notable difference between most ITP patients and the GT patient is that the GT patient had a high titer of GPIIbIIIa alloantibodies in the serum whereas ITP patients rarely have free autoantibodies (not bound to platelets) in the serum. This could be because the autoantibodies in ITP patients are sequestered by platelets and are quickly removed from the system, reducing the concentration of autoantibodies in circulation. Whereas, the alloantibodies in the GT patient were not removed from circulation since the alloantibodies do not bind to their own platelets and the transfused platelets have been removed from the system. This strong humoral memory to GPIIbIIIa suggests that the GT patient is likely refractory to platelet transfusions[10]. Our group also tested a patient with BSS since their platelets have quantitative and/or qualitative defects of GPIbIX. Anti-GPIbIX plasma cells and memory B cells were not detected in the BSS patient, and unlike the GT patient, the BSS patient did not have anti-GPIbIX antibodies in the serum. This patient has also never received platelet transfusions previously, which explains why anti-GPIbIX alloantibodies and alloreactive ASCs were not detected[94]. Future experiments could involve testing GT patients, BSS patients and ITP patients with varying titers of free

antibodies in the serum to determine whether there is a correlation between the titer of antibodies and the frequency of autoreactive B cells.

In another attempt to enhance detection of autoreactive cells in ITP patients, we stimulated mononuclear cells with R848, IL-2, and IL-21. This is because a significantly increased level of IL-21 has been detected in the peripheral blood of patients with autoimmune diseases such as SLE, RA, celiac disease, type I diabetes, multiple sclerosis, and ITP[85,86,95]. IL-21 has also been associated with autoantibody production and therapeutic agents that block IL-21 signaling are undergoing clinical trials and have already shown to reduce autoantibodies. Although stimulating PBMCs with R848, IL-2, and IL-21 significantly increased the number of total IgG ASCs compared to stimulating PBMCs with R848 and IL-2 alone, it did not enhance the detection of anti-GPIIbIIIa and anti-GPIbIX ASCs. In contrast, there was a significant increase in anti-GPIIbIIIa antibodies in the supernatants of the cells stimulated with R848, IL-2 and IL-21 compared to the supernatants of the cells stimulated with only R848 and IL-2. It is possible that IL-21 did activate autoreactive B cells, but these cells could have died before day 6 of stimulation and would not have been detected in the ELISPOT assay. Supernatants from PBMCs stimulated with IL-21 from healthy donors should be tested to determine whether the presence of IL-21 increases the concentration of anti-GPIIbIIIa antibodies in this negative control group. IL-21 is a regulatory cytokine that is mainly secreted by activated CD4+ T cells and is capable of inducing naïve and memory B cells to differentiate into plasma cells[86]. Although IL-21 is associated with autoimmunity, IL-21 is pleiotropic and effects various types of cells and signaling mechanisms. Additionally, stimulation of autoreactive

B cells may require IL-21 along with other factors present in the environment of autoimmune diseases to function collectively and eventually have downstream effects for autoantibody production[85].

Another method of enhancing antigen-specific ASCs is by stimulating memory B cells with the antigen of interest. In a study, Dsg3 was used in addition to polyclonal stimulants to stimulate PBMCs from patients to detect autoreactive memory B cells[91]. 1.3-2.3 Dsg3 specific ASCs/10^5 PBMCs were detected in unstimulated PBMCs only from patients with severe conditions. When PBMCs were stimulated with Dsg3, 6.3-84.0 Dsg3 ASCs/10^5 PBMCs were detected even in patients with mild conditions, which increases the diagnostic sensitivity. In our experiments, stimulation of PBMCs with R848 and IL-2 along with the addition of purified GPIIbIIIa and GPIbIX caused the cells to aggregate, preventing them from being used in the ELISPOT assay (data not shown). Further optimization of the duration of antigen stimulation and the concentration of antigens used to stimulate the cells needs to be determined. Additionally, it is highly likely that the PBMCs isolated by density gradient centrifugation were contaminated with platelets, which contains the antigen of interest for ITP patients[96]. As the PBMCs were stimulated with R848 and IL-2, contaminating platelets in the PBMCs could have stimulated autoreactive memory B cells in the PBMCs of ITP patients. Another opportunity for the cells to be stimulated directly by the antigen was when PBMCs were incubated in the ELISPOT plate wells coated with the immobilized GPIIbIIIa and GPIbIX. However, the lack of antigen-specific spots indicates that these methods of antigen stimulation were not successful. Unlike the antigens on platelets, the purified proteins may have changed the conformation

of the protein complexes once immobilized on the ELISPOT plate, preventing the autoreactive B cells from binding to certain epitopes on the antigen and inducing differentiation of the autoreactive B cells into plasma cells. In a study when CD4+ T cells from ITP patients were incubated with purified GPIIbIIIa, the T cells did not proliferate, and autoantibody production was not enhanced[97]. Whereas, stimulating the cells with a fragment of GPIIbα (18-259) resulted in the proliferation of autoreactive T cells and this was also associated with enhanced autoantibody production. T cells in ITP patients are known to recognize cryptic sites on GPIIbIIIa[98]. Our lab had also shown that the highest number of ASCs specific to platelet factor 4 (PF4)/Heparin complexes were produced when PBMCs were stimulated with R848, IL-2, and PF4/Heparin (unpublished data). In another study, autoantibody production was also enhanced in patients with systemic sclerosis when the PBMCs were stimulated with the antigen, DNA topoisomerase I[99]. The B cell – T cell interaction is essential for B cell activation, differentiation, proliferation, affinity maturation, and antibody production[42]. This was also indicated in our experiment when the B cells that were isolated from stimulated PBMCs resulted in detection of a higher number of total IgG ASCs than the B cells that were isolated and then stimulated likely due to the B cell – T cell interactions in PBMCs. Future experiments should optimize stimulation of both autoreactive T cells and B cells with fragments of GPIIbIIIa and GPIbIX in addition to R848 and IL-2.

5.2. Autoantibodies and Autoreactive B Cells in the Bone Marrow and the Peripheral Blood

Rheumatoid arthritis, multiple sclerosis, and type I diabetes are all examples of diseases known to have niche restricted autoimmunity, where the disease activity occurs in a specific compartment[79–81]. For example in ITP, the spleen is an ideal compartment for accelerated platelet destruction since the spleen is where anti-platelet autoantibodies are produced, where macrophages reside, and where platelets are sequestered[27,68,100]. Platelet underproduction in ITP patients indicates that the bone marrow is another compartment involved in the pathophysiology of ITP[17,101]. Sequestration of autoantibodies in the bone marrow is one of the potential explanations for the lack of autoantibodies detected in the peripheral circulation of 50-60% of the ITP patients. Although multiple studies have hypothesized that autoantibodies impair platelet production, autoantibodies have yet to be detected in the bone marrow. Thus, we examined the bone marrow and the peripheral blood compartments of 12 ITP patients, 3 non-immune thrombocytopenic patient controls and 5 healthy controls for the presence of anti-GPIIbIIIa and anti-GPIbIX IgG autoantibodies and autoreactive B cells.

For the first time, we detected autoantibodies with known specificity to GPIIbIIIa and GPIbIX in the bone marrow of a subset of ITP patients. The diagnostic sensitivity of detecting autoantibodies in the bone marrow compartment of ITP patient (42%) was the same as detecting autoantibodies in the peripheral blood compartment (42%). However, examining both compartments for autoantibodies increased the diagnostic sensitivity (58%). The diagnostic specificity of detecting autoantibodies in the bone marrow and the

peripheral blood was high (100%) since autoantibodies were not detected in any of the non-immune thrombocytopenic patient controls or the healthy controls. In previous studies, the extent of detecting autoantibodies in the bone marrow of ITP patients was the detection of increased levels of IgG antibodies of unknown specificity on the surface of megakaryocytes[40]. However, this finding was not exclusive to immune-mediated thrombocytopenia since an increased level of IgG antibodies were also detected on the surface of megakaryocytes from non-immune thrombocytopenic patients.

In our findings, there was no association between the presence or absence of autoantibodies in the bone marrow and the peripheral blood compartments of ITP patients. Autoantibodies were present at high concentrations in the periphery, whereas the concentrations of autoantibodies were lower in the bone marrow, except for one ITP patient who had a high concentration of anti-GPIIbIIIa autoantibodies. Autoantibody detection in the bone marrow may have been limited by the low frequency of GPIIbIIIa and GPIbIX expressing cells in the bone marrow. In our study, the platelet count was lower in the bone marrow than the peripheral blood, and the frequency of megakaryocytes are usually < 1% of myeloid cells in the bone marrow[102]. It is also possible that autoantibodies bound to platelets and/or megakaryocytes in the bone marrow could have been already destroyed by macrophages, limiting the detection of cell-bound autoantibodies. This is a similar limitation encountered when platelet-bound autoantibodies are detected in the peripheral blood of ITP patients with a low platelet count.

We also identified specific subsets of ITP patients based on the autoantibody profile in the bone marrow and the peripheral blood of each patient. We did not observe a general

trend in the presence or absence of autoantibodies in either of the compartments. A subset of patients had autoantibodies in both compartments, another subset of patients did not have autoantibodies in either of the compartments, while the remaining patients had autoantibodies in only one of the compartments. The patients without detectable autoantibodies in either of the compartments suggests that mechanisms independent of autoantibodies are involved in ITP. One such mechanism includes cytotoxic T cell-mediated platelet lysis[103]. It is also possible that autoantibodies of other classes and/or autoantibodies with specificity to other antigens that were not tested in our study may be involved in the pathophysiology of ITP. Although the role of autoantibodies in the bone marrow have yet to be studied, the presence of autoantibodies in the bone marrow contributes to the hypothesis that autoantibodies might be sequestered in the bone marrow and may impair platelet production. Whereas, the autoantibodies detected in the peripheral blood are likely involved in autoantibody-mediated platelet destruction. Furthermore, the presence of autoantibodies in both of the compartments suggests that the autoantibodies may have a dual function of opsonizing platelets for destruction and also impairing platelet production in some ITP patients. This is because antigens such as GPIIbIIIa and GPIbIX are expressed on both platelets and megakaryocytes. Of the 7 ITP patients who did not have autoantibodies in the peripheral blood, only 2 patients had autoantibodies in the bone marrow, indicating that the lack of autoantibodies detected in the peripheral blood is not necessarily due to sequestration of autoantibodies in the bone marrow. When we compared the autoantibody profiles of 4 ITP patients, who had splenectomy and 4 ITP patients who did not have a splenectomy, general trends in the autoantibody profile were not observed

between the two groups. Each patient had a distinct autoantibody profile indicating that other characteristics of the patients such as treatment (a type of medications) should be incorporated in these studies. Furthermore, the patient who had splenectomy and also had autoantibodies in the periphery but did not have autoantibodies in the bone marrow suggests that there might be other sites of autoantibody production aside from the spleen and the bone marrow.

Autoantibodies have also been detected in the bone marrow of patients with other autoimmune diseases. In SLE patients, autoantibodies were detected on BMMCs and were also shown to correlate with the presence of anti-nuclear autoantibodies in the peripheral blood[104]. Autoantibodies have also been detected in the bone marrow of patients with immune-related pancytopenia[105]. These autoantibodies are known to suppress hematopoiesis by binding to BMMCs and activate macrophages for phagocytosis of the cells. Additionally, bone marrow biopsy studies of ITP patients showed monocytes near megakaryocytes as some megakaryocytes were being phagocytosed[38,43]. Evidence of phagocytosis in the bone marrow along with the presence of GPIIbIIIa and/or GPIbIX autoantibodies further contributes to the hypothesis that autoantibodies are sequestered in the bone marrow.

Examining the bone marrow for ASCs showed that many total IgG plasma cells and plasmablasts were present in the bone marrow, whereas total IgG ASCs were rarely detectable in the peripheral blood. This is because the bone marrow is where long-lived plasma cells reside, whereas the peripheral blood mostly consists of naïve and memory B cells[28,106]. Detection of large spots formed by ASCs in unstimulated BMMCs indicates that

the plasma cells secrete a high quantity of antibodies in vivo. Anti-GPIIbIIIa plasma cells were detected in the bone marrow of some of the ITP patients and healthy control. After BMMCs were stimulated, anti-GPIIbIIIa ASCs were detected in some of the ITP patients and a non-immune thrombocytopenic patient. Anti-GPIbIX ASCs were also detected in a non-immune thrombocytopenic patient after stimulation. Since plasma cells are terminally differentiated cells, these cells cannot proliferate, so any increase in the number of IgG ASCs after stimulation of BMMCs suggests that memory B cells are present in the bone marrow. However, it is also possible that the memory B cells detected after stimulation could have been the memory B cells from the peripheral blood that contaminated the bone marrow aspirate sample during sample collection. The spots detected in unstimulated BMMCs are long-lived plasma cells, and it is possible that spots detected after BMMCs stimulation could be produced by those long-lived plasma cells that survived the stimulation process. In a study, R848 was shown to increase IL-6 secretion in mouse BMMCs, which is one of the factors required to maintain long-term survival of long-lived plasma cells[107]. However, future experiments should assess whether memory B cells are present in the bone marrow and whether long-lived plasma cells can survive the BMMCs stimulation process.

Our findings indicate that anti-GPIIbIIIa and anti-GPIbIX autoreactive long-lived plasma cells are present at low frequencies in the bone marrow compartment in only a small subset of ITP patients, but were also detected in some of the thrombocytopenic patients and healthy controls. Anti-GPIIbIIIa plasma cells were detected in unstimulated PBMCs of some ITP patients, a non-immune thrombocytopenic patient, and a healthy control and

GPIbIX specific IgG ASCs were detected in the unstimulated PBMCs of some ITP patients. Whereas, antigen-specific ASCs were not detected in stimulated PBMCs. These findings indicate that autoreactive plasma cells were present in the circulation and that autoreactive memory B cells were not present. The frequencies of antigen-specific ASCs detected in ITP patients were low (<0.5 ASCs/10^5 PBMCs), and the sample size varies among the groups, resulting in a lack of signal separation between the ITP patients, the non-immune thrombocytopenic control and the healthy control groups. The frequencies of antigen-specific ASCs in the peripheral blood of ITP patients was higher in other studies compared to our findings as mentioned earlier[66,67]. Additionally, those studies also had detectable antigen-specific ASCs in the non-immune thrombocytopenic patients (1.60 ± 3.00 GPIIbIIIa IgG ASCs/10^5 PBMCs), and the healthy donors (0.40 ± 0.30 GPIIbIIIa IgG ASCs/10^5 PBMCs), which are comparable to the frequencies of ASCs detected in the control groups in our study [66]. In another study, anti-GPIIbIIIa antibodies were also shown to correlate with the frequency of GPIIbIIIa ASCs in the spleen and the peripheral blood[30]. There was no relationship between detection of autoantibodies and the presence of antigen-specific ASCs in the bone marrow or the peripheral blood.

Overall, detecting anti-GPIbIX ASCs had a higher specificity than detecting anti-GPIIbIIIa ASCs in both the bone marrow and the peripheral blood compartments since anti-GPIIbIIIa ASCs were detected in the control groups. In contrast, detecting anti-GPIIbIIIa ASCs had a higher sensitivity than detecting GPIbIX ASCs in both compartments. The sensitivity of detecting autoreactive ASCs before and after stimulation of mononuclear cells was the same in the bone marrow (42%) compared to the autoreactive

ASCs detected in the peripheral blood (42%). However, combining the detection of anti-GPIIbIIIa or anti-GPIbIX autoreactive ASCs in either of the compartments increased the sensitivity (58%). Although the sensitivities of detecting ASCs are similar to the sensitivities of detecting autoantibodies in each compartment, the specificity of detecting autoreactive ASCs is lower than the specificity of detecting autoantibodies in both of the compartments. Cut-off values for the ELISPOT assay needs to be established and applied to the results to determine a more accurate sensitivity and specificity of detecting autoreactive ASCs. Additionally, there was no association between the presence of autoantibodies and autoreactive B cells in either one of the compartments of the ITP patients.

5.3. Limitations

One of the possible limitations of the ELISPOT assay is that immobilization of the antigens on the PVDF membrane could have changed the conformation of the protein complexes and prevented autoantibodies secreted by ASCs from binding to the pathogenic epitopes. Although spots formed by Raj-1 and TW-1 hybridoma cells were detected, changes in the confirmation of the immobilized antigens may not have altered the epitopes of these antibodies. A solution to resolve this problem would be to avoid immobilizing the antigens by using soluble biotinylated GPIIbIIIa and GPIbIX in variation 2 of the ELISPOT assay (Figure 20). However, this would only be useful for detecting IgM antigen-specific ASCs since non-specific binding of IgM antibodies would occur when attempting to detect IgG and IgA antigen-specific ASCs as discussed earlier. Another possibility for the lack of antigen-specific ASCs detected in ITP patients is that low affinities autoantibodies could have been washed out during the wash steps involved in the ELISPOT assay and spots may not have been formed. This is because B cells with low affinity to the self-antigens are the cells that are more likely to escape tolerance mechanisms rather than the B cells with high affinity to the self-antigens[78]. Additionally, antibodies specific to the cytoplasmic domain of GPIIbIIIa and GPIbIX are known to exist, so it is possible that some of the spots detected in the antigen-specific wells were not autoreactive ASCs relevant to the pathogenesis of ITP[98]. This may explain why some of the healthy donors had anti-GPIIbIIIa and anti-GPIbIX ASCs. The immunodominant epitopes relevant to the pathogenesis of ITP are known to exist on the extracellular domain of the protein complexes[98].

When the ELISPOT assay was conducted using unstimulated BMMCs, non-specific spots were observed in the antigen-coated wells and the no coat negative control wells (wells without coating antigens or antibodies). When BMMCs were cultured for 6 days with stimulants, nonspecific spots were no longer present, but BMMCs that were cultured for 6 days without stimulants still produced non-specific spots. This suggests that the stimulants caused the nonspecific spot forming cells to die when the cells were cultured for 6 days. To obtain our results, nonspecific spots in the no coat wells were subtracted from the spots detected in the antigen-coated wells. However, future experiments should eliminate binding of the non-specific spots and reduce the background signal. Similarly, non-specific spots produced by BMMCs were also observed in a study detecting vaccinia-specific IgG long-lived plasma cells in human bone marrow samples[106]. However, these non-specific spots were not addressed in the study. Unlike our study, the authors used wells coated with the antigen of interest without incubation of cells as the negative control rather than incubating the cells in the no coat wells. Another possible limitation of the study is that when the bone marrow aspirate samples were obtained, the samples may have been contaminated with peripheral blood. This could explain why we observed a positive correlation between the platelet count in the bone marrow aspirate and the peripheral blood (data not shown). In other studies, peripheral blood dilution has been assessed by comparing hematocrit levels in the blood and the aspirate, which should be incorporated in future studies[108]. Furthermore, the bone marrow aspirate samples were filtered through a 70 µm filter to remove any bone fragments before BMMCs were isolated. There is a possibility that megakaryocytes might have been filtered out due to size since

megakaryocytes can reach up to 100 μm or the megakaryocytes could have been attached to the bone fragments. Thus, autoantibodies bound to GPIIbIIIa and/or GPIbIX on megakaryocytes could have been missed when testing for autoantibodies in the AC assay.

Contaminating platelets are known to die and disintegrate when the cells are incubated at 37°C[109]. It is possible that autoantibodies were not detected in the cell culture supernatants using the indirect ELISA because the secreted anti-GPIIbIIIa and anti-GPIbIX autoantibodies may have bound to the soluble GPIIbIIIa and GPIbIX from disintegrated platelets in the PBMCs cultures. Due to the technical steps involved in the indirect ELISA used to detect autoantibodies in the supernatants, the assay is only capable of detecting free autoantibodies, so autoantibodies bound to antigens are not detected. To overcome this challenge, ELISA plate wells could be coated with Raj-1 and TW-1 antibodies and supernatants can be incubated in these wells along with the addition of purified GPIIbIIIa and/or GPIbIX. This would allow autoantibodies in the supernatant to bind to the purified GPIIbIIIa and GPIbIX and along with the autoantibodies already bound to the antigens from disintegrated platelets to be detected by Raj-1 and TW-1 antibodies on the ELISA plate. The indirect ELISA also involves wash steps, so autoantibodies of low affinity would not be detected. Antibodies in the supernatants should also be concentrated to enhance detection of autoantibodies. These are some of the limitations involved in our study and should be considered when assessing the results.

6. FUTURE DIRECTIONS:

One of the initial steps is to increase the sample size of the bone marrow and the peripheral blood samples tested from ITP patients, non-immune thrombocytopenic patient controls and healthy controls. This is especially important when testing for autoreactive ASCs in the ELISPOT assay to determine whether a greater separation of the signal can be obtained and also to establish cut-offs. ITP patients who have not received treatment should be studied since the treatment options may impact the detection of autoantibodies and autoreactive ASCs. For future experiments, PBMCs should be stimulated with R848 and IL-2 along with the fragments GPIIbIIIa and GPIbIX to stimulate autoreactive T cells, which may enhance proliferation of autoreactive B cells. After stimulation of the PBMCs, the B cells can be isolated and used in the ELISPOT assay to maximize the concentration of antigen-specific ASCs in the ELISPOT wells. Another future study could be to determine whether the increased amount of autoantibodies detected in the bone marrow is associated with the amount of antibodies detected on the surface of megakaryocytes. Comparison of autoantibodies and ASCs in the bone marrow, peripheral circulation, and the spleen may provide a more in-depth insight into the mechanisms involved in certain subsets of ITP patients. The presence of autoantibodies and autoreactive ASCs have been associated with predicting disease flare-ups or disease activity in SLE[93]. Thus, testing for autoantibodies and autoreactive ASCs in the bone marrow and the peripheral blood at multiple time points of during the course of the disease may provide more insight into the severity of thrombocytopenia in ITP patients. In addition, ITP patients who have cyclical

platelet count or those with cyclical thrombocytopenia would be another patient group of interest to assess autoantibodies and autoreactive ASCs to understand disease activity.

Even though IgM and IgA autoantibodies are present in low titers in the peripheral circulation of few ITP patients, the presence and the function of IgM and IgA autoantibodies and autoreactive ASCs that secrete IgM and IgA autoantibodies might differ in the bone marrow. Thus, IgM and IgA autoantibodies should be investigated in the bone marrow in future studies. When detecting IgG, IgA and IgM ASCs, we proposed using 2 variations of the ELISPOT assay (Figure 20). To circumvent the non-specific binding of IgM antibodies, specific binding of IgG and IgA antibodies can be determined by using the antigen to coat ELISPOT wells and capture antibodies secreted by ASCs (Figure 20a). To detect specific IgM spots, biotinylated antigens can be used to detect antibodies that were secreted by IgM ASCs (Figure 20b). More sensitive autoantibody detection tools should also be used to detect low-affinity autoantibodies. More recent findings have suggested that antibodies targeting TPO, TPO receptor (cMpl) and/or the TPO/cMpl complex may inhibit megakaryopoiesis[110]. Purified cMpl autoantibodies from ITP patient sera were also shown to inhibit megakaryopoiesis[111]. In future experiments, the presence of autoantibodies and autoreactive ASC specific to other antigens such as TPO, cMpl, TPO/cMpl complex should be tested especially since TPO is known to regulate platelet production[2]. Lastly, megakaryocytes could be cultured with autoantibodies purified from the bone marrow, or with the bone marrow fluid to determine whether the autoantibodies interfere with megakaryopoiesis/ thrombopoiesis.

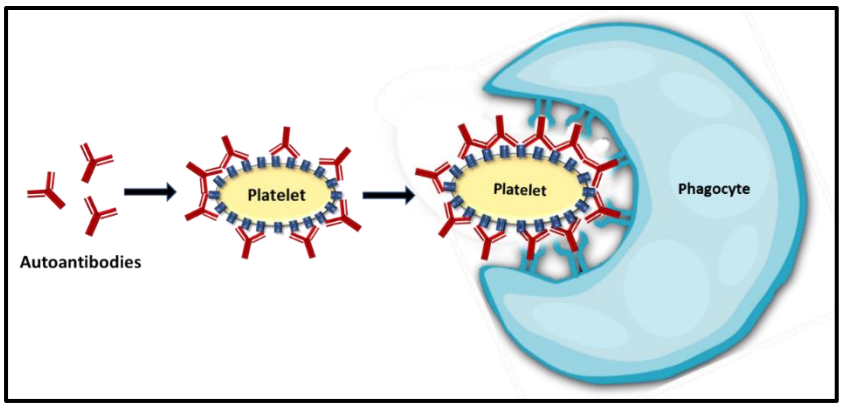
7. CONCLUSION:

In this study, we optimized an ELISPOT assay to detect anti-GPIIbIIIa and anti-GPIbIX autoreactive IgG ASCs in ITP patients using hybridoma cells. As a proof of principle, we showed that a GT patient with a high titer of anti-GPIIbIIIa alloantibodies in circulation had a high frequency of anti-GPIIbIIIa alloreactive memory B cells in the peripheral blood. However, autoreactive plasma cells and memory B cells were rarely detectable in the peripheral blood of ITP patients. Furthermore, testing for autoreactive B cells in only one of the peripheral blood or the bone marrow compartment of ITP patients had low sensitivity and low specificity. Larger sample size is required to determine whether a greater separation of the signal can be achieved between the ITP patients and the control groups to establish cut-offs for the ELISPOT assay. Furthermore, detecting autoantibodies in the bone marrow and the peripheral blood separately diagnostic sensitivity of 42% for each compartment. Whereas, testing for autoantibodies in both of the compartments increased the diagnostic sensitivity to 58% while maintaining a specificity of 100%. Majority of the ITP patients without detectable autoantibodies in the peripheral blood did not have detectable autoantibodies in the bone marrow, which suggests that autoantibodies are not sequestered in the bone marrow of this subset of patients. Additionally, there was no correlation between the presence of autoantibodies and ASCs in the bone marrow and the peripheral blood. In conclusion, we detected autoantibodies and autoreactive B cells of known specificity for the first time in the bone marrow of a subset of ITP patients. We also showed that the sensitivity of detecting autoantibodies or autoreactive B cells separately was the same in the bone marrow compared to the peripheral blood. Whereas, examining

both compartments increased the diagnostic sensitivity. Our results further indicate that as a biomarker in ITP, testing for autoantibodies using the direct AC assay is better than testing for autoreactive B cells using the ELISPOT assay due to the higher diagnostic specificity of autoantibody testing.

8. FIGURES:

a)



b)

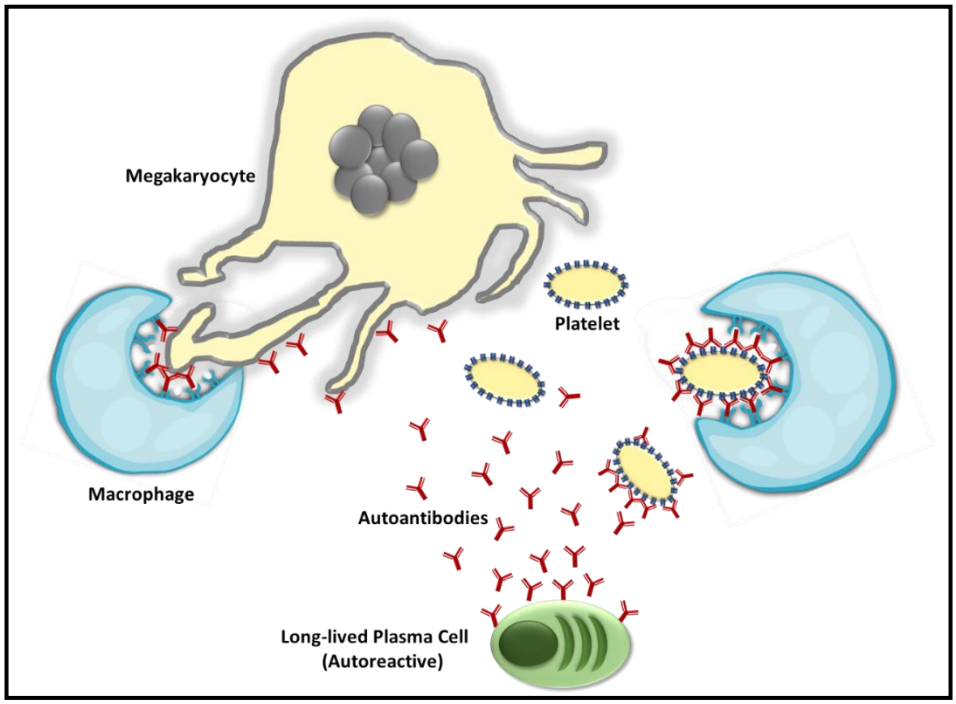
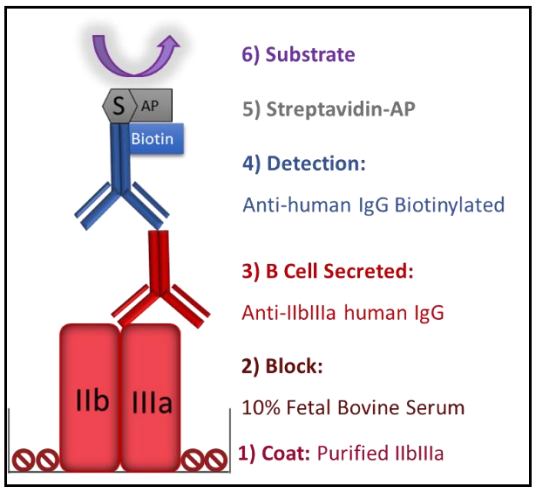
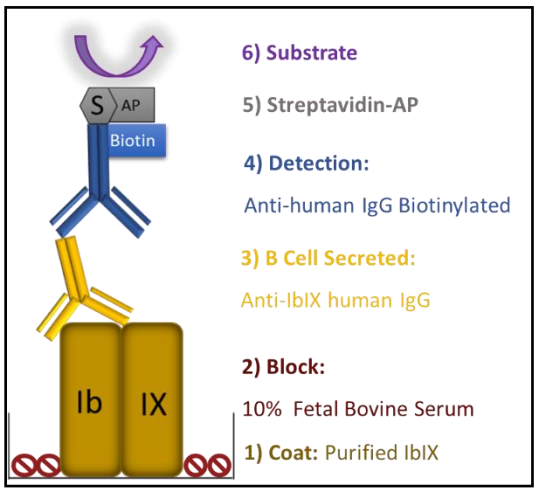


Figure 1. **Schematic diagram of autoantibody-mediated mechanisms involved in ITP.** (a) Autoantibody-mediated platelet destruction. Autoantibodies bind to antigens on platelets and target the cells for destruction through Fc-Fc receptor interactions with phagocytic cells. (b) Autoantibody-mediated platelet underproduction in the bone marrow. Autoantibodies bind to antigens on megakaryocytes and interfere with megakaryopoiesis and/or thrombopoiesis.

a)



b)



c)

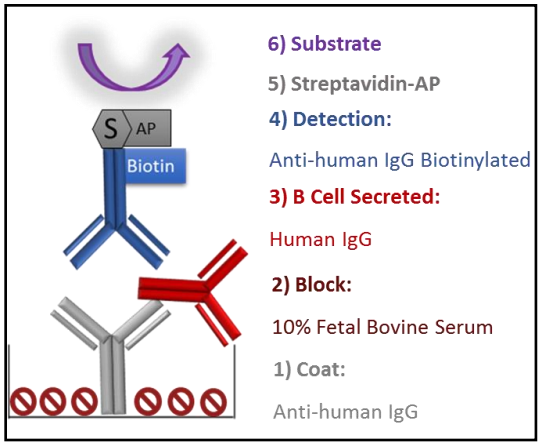


Figure 2. Schematic diagram of steps involved in detecting antigen-specific and total IgG ASCs in the ELISPOT assay. A single ASC secretes antibodies that bind to the coating antigens or antibodies immobilized on the ELISPOT plate wells. Detection of secreted antibodies collectively forms a spot representing a single ASC. Details of the steps are described in the materials and methods section.

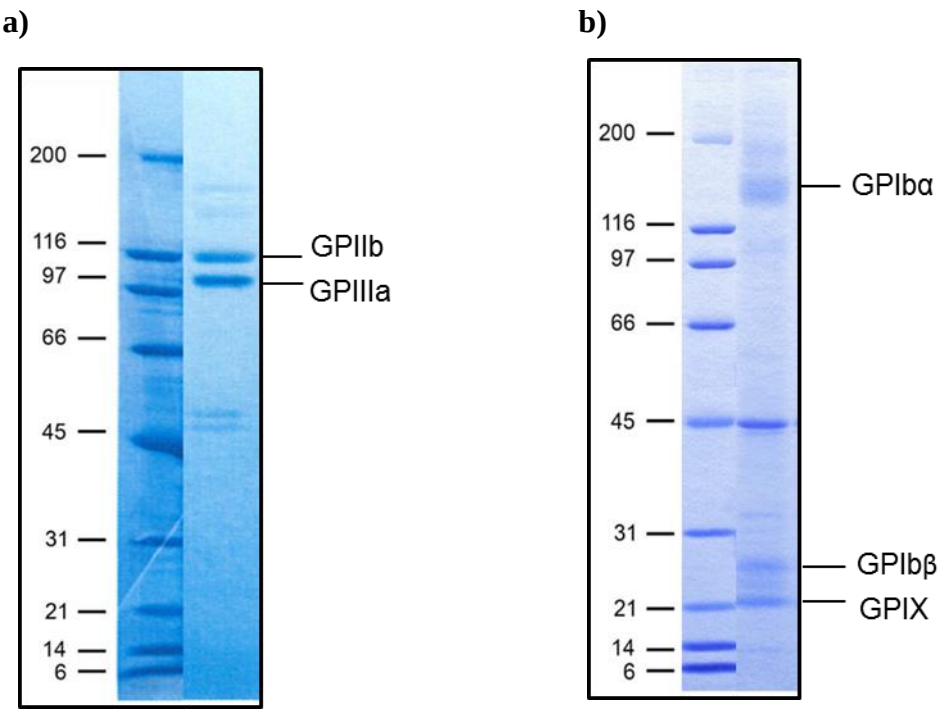
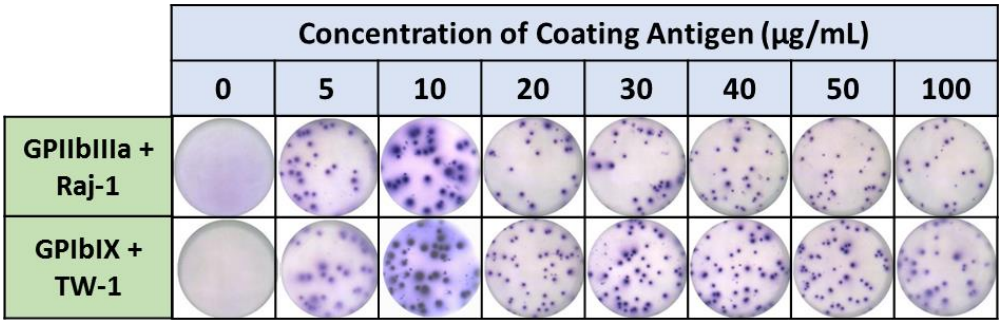
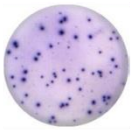
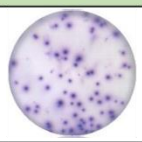
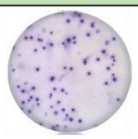


Figure 3: SDS-PAGE analysis of affinity purified GPIIbIIIa and GPIbIX from human platelets. The left lane consists of the molecular weight markers alongside the eluates containing purified platelet (a) GPIIbIIIa and (b) GPIbIX on the right lane.

a)



b)

Coating	GPIIbIIIa	Anti-mouse IgG	No Coat
Cells	Raj-1	Raj-1	Raj-1
Wells			
Count	73 ± 11	74 ± 10	0 ± 0
Coating	GPIbIX	Anti-mouse IgG	No Coat
Cells	TW-1	TW-1	TW-1
Wells			
Count	91 ± 10	95 ± 10	0 ± 0

c)

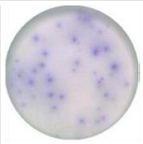
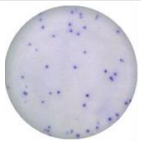
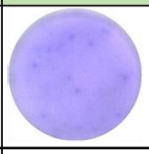
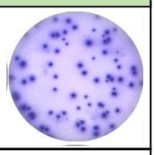
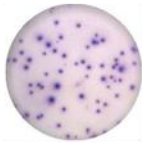
Coating	GPIIbIIIa	Anti-mouse IgG
Cells (4 hr)	Raj-1	Raj-1
Wells		
Count	28 ± 9	52 ± 5
Cells (Overnight)	Raj-1	Raj-1
Wells		
Count	5 ± 4	72 ± 2

Figure 4: Validation of the ELISPOT assay using Raj-1 and TW-1 hybridoma cells. (a) Raj-1 and TW-1 hybridoma cells were incubated overnight in wells coated with 0 µg/mL- 100 µg/mL of GPIIbIIIa and GPIbIX respectively. (b) Approximately 100 Raj-1 and 100 TW-1 hybridoma cells were incubated in wells coated with 30 µg/mL of GPIIbIIIa, 30 µg/mL of GPIbIX, anti-mouse IgG antibodies (positive control) and wells without coating antigens or antibodies (negative control) overnight. (c) 100 Raj-1 cells were incubated in wells coated with 30 µg/mL of partially degraded GPIIbIIIa for 4-hours and an overnight. Results are indicated as mean ± 1 SD of 4 replicates.

Coating	GPIIbIIIa	GPIIbIIIa	GPIIbIIIa	No Coat
Cells	Raj-1	Raj-1 + Platelets	Raj-1 + PBMCs	Raj-1
Wells				
Count	62 ± 6	63 ± 5	74 ± 4	0 ± 0

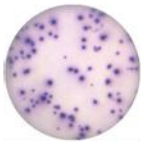
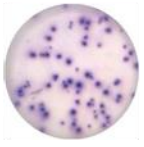
Coating	GPIbIX	GPIbIX	GPIbIX	No Coat
Cells	TW-1	TW-1 + Platelets	TW-1 + PBMCs	TW-1
Wells				
Count	91 ± 2	89 ± 17	100 ± 6	0 ± 0

Figure 5: Detection of spots formed by Raj-1 and TW-1 hybridoma cells in the presence of platelets and PBMCs. 100 Raj-1 and 100 TW-1 hybridoma cells were incubated in wells coated with GPIIbIIIa and GPIbIX, respectively and also in no coat wells (negative control) overnight. Hybridoma cells were also incubated in the wells with 400 000 platelets and with 400 000 PBMCs. Results are indicated as mean ± 1 SD of 4 replicates.

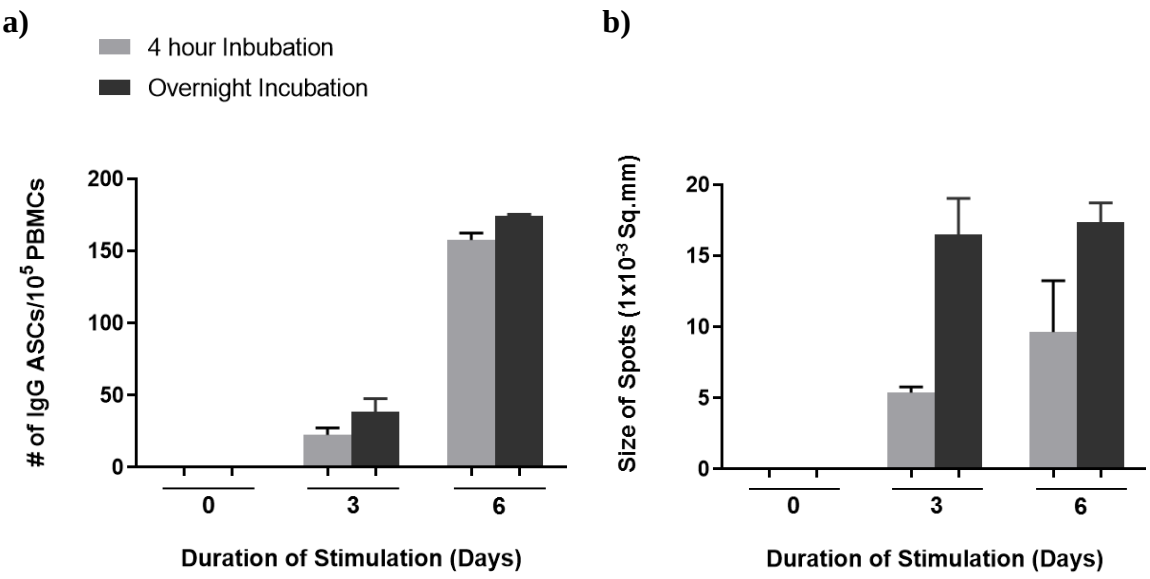
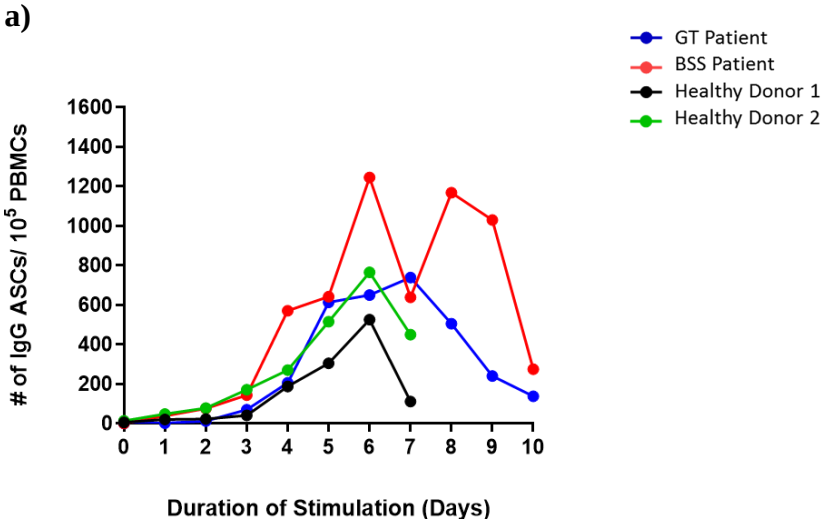
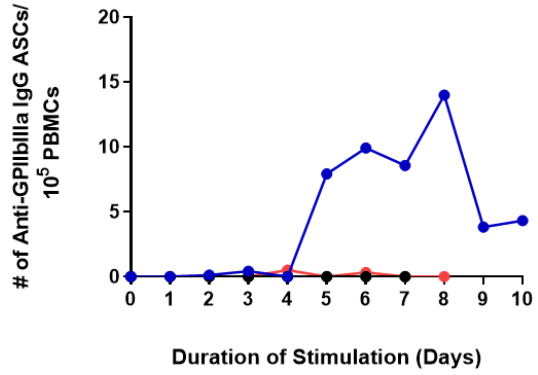


Figure 6: Quantitative analysis of spots formed by total IgG ASCs in unstimulated and stimulated PBMCs. PBMCs were incubated in the ELISPOT plate wells for 4 hours and overnight on day 0 (unstimulated), and after stimulating PBMCs with R848 and IL-2 on day 3 and day 6 of stimulation. a) The number of spots and (b) the size of spots detected in each condition is represented as mean ± 1 SD of 4 replicates.

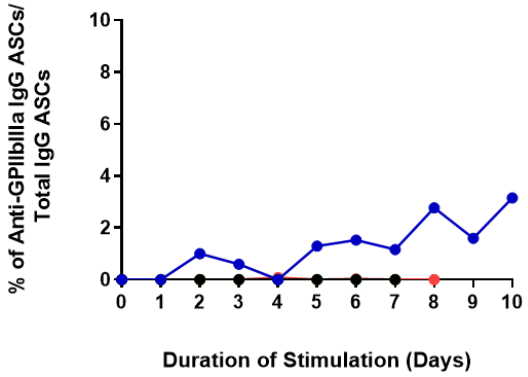
a)


GT Patient
BSS Patient
Healthy Donor 1
Healthy Donor 2
# of IgG ASCs/ 10^5 PBMCs
Duration of Stimulation (Days)

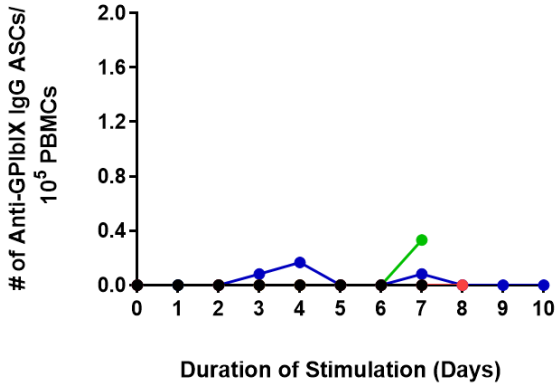
b)


# of Anti-GPIIbIIIa IgG ASCs/ 10^5 PBMCs
Duration of Stimulation (Days)

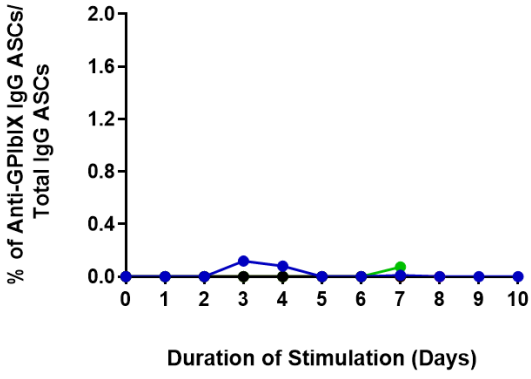
c)


% of Anti-GPIIbIIIa IgG ASCs/ Total IgG ASCs
Duration of Stimulation (Days)

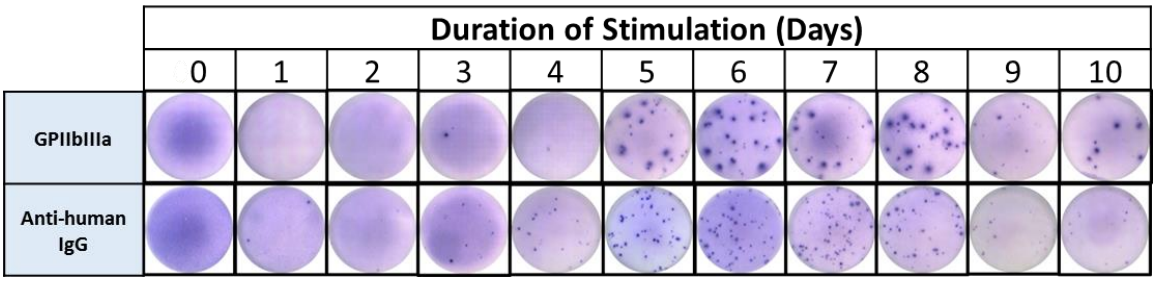
d)


# of Anti-GPIbIX IgG ASCs/ 10^5 PBMCs
Duration of Stimulation (Days)

e)


% of Anti-GPIbIX IgG ASCs/ Total IgG ASCs
Duration of Stimulation (Days)

f)



g)

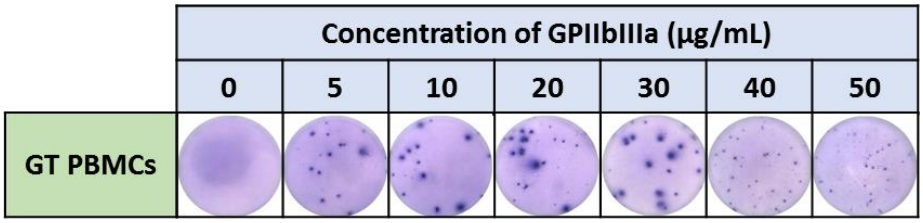


Figure 7: Time course detecting total IgG and antigen-specific ASCs during PBMCs stimulation. Anti-GPIIbIIIa, anti-GPIbIX and total IgG ASCs were detected daily in PBMCs samples from day 0 (unstimulated) to day 7 (n=2) or day 10 (n=2) of stimulation. (a) Detection of total IgG ASCs is represented as the number of spots in 100 000 PBMCs. (b,d) Detection of anti-GPIIbIIIa and anti-GPIbIX IgG ASCs is represented as the number of spots in 100 000 PBMCs and (c,e) as a relative frequency of total IgG ASCs. (f) Representative wells display anti-GPIIbIIIa IgG spots and total IgG spots detected in the GT patient's PBMCs during the PBMCs stimulation time course. Anti-GPIIbIIIa spots were produced from 300 000 PBMCs and total IgG spots were produced from 10 000 PBMCs. (g) Representative wells display spots produced by the GT patient's PBMCs on day 6 of stimulation in wells coated with 0 µg/mL - 50µg/mL of GPIIbIIIa.

a) **b)**

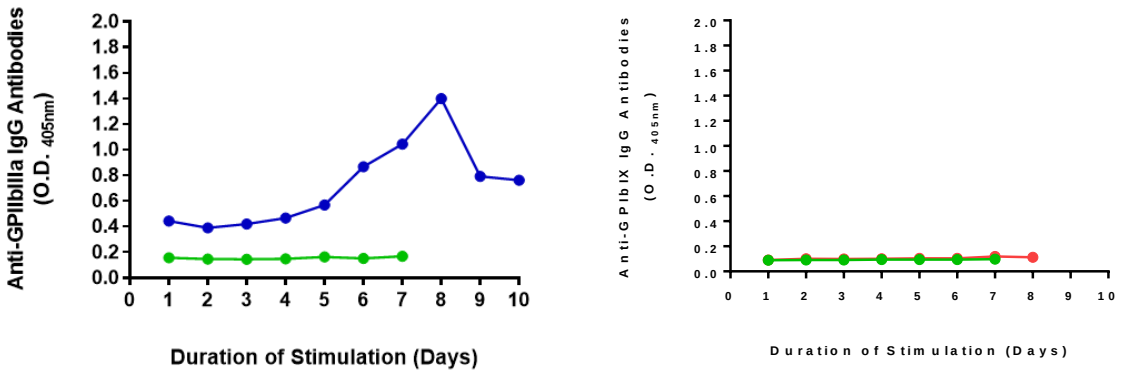
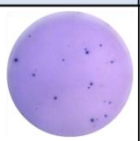
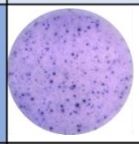
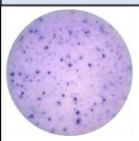
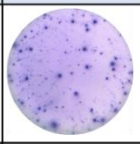
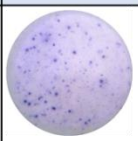
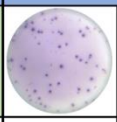
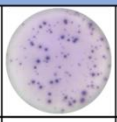
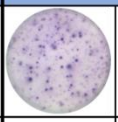
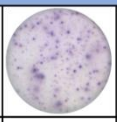
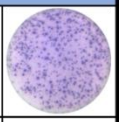


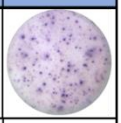
Figure 8: Time course detecting antigen-specific IgG antibodies in the supernatants of stimulated PBMCs. The media supernatants of the PBMCs cultured during stimulation from day 1 to day 10 were tested for the presence of antigen-specific IgG antibodies. The indirect ELISA was used to test (a) supernatants from the GT patient and a healthy donor for the presence of anti-GPIIbIIIa IgG antibodies and to test (b) supernatants from the BSS patient and a healthy donor for the presence of anti-GPIbIX IgG antibodies.

a)

Coating	GPIIbIIIa	GPIbIX	IgG	No Coat	No Cells
Biotinylated Anti-human IgG					
Count (/10^5 PBMCs)	0 ± 0	0 ± 0	180 ± 45	0 ± 0	0 ± 0
Coating	**GPIIbIIIa**	**GPIbIX**	**IgM**	**No Coat**	**No Cells**
Biotinylated Anti-human IgM					
Count (/10^5 PBMCs)	95 ± 6	58 ± 7	2190 ± 151	1060 ± 208	0 ± 0

b)

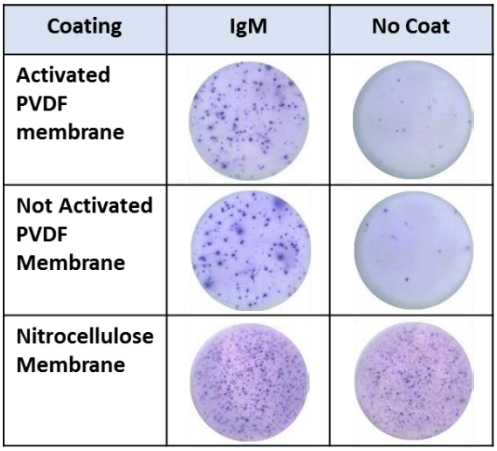
Coating	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM
Detection	IgA			IgG			IgM		
10 000 PBMCs									
Count	44 ± 9	0 ± 0	0 ± 0	0 ± 0	80 ± 15	0 ± 0	72 ± 11	48 ± 11	270 ± 25

Coating	No Coat		
Detection	IgA	IgG	IgM
10 000 PBMCs			
Count	0 ± 0	1 ± 1	83 ± 11

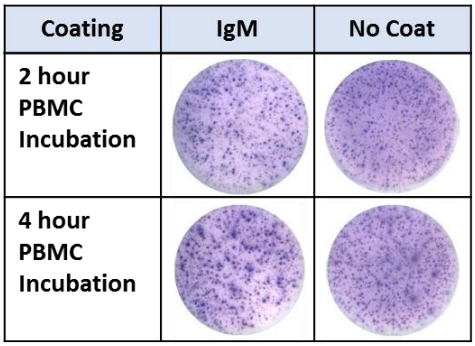
Coating	IgA	IgG	IgM
Detection	IgA	IgG	IgM
0 PBMCs			
Count	0 ± 0	0 ± 0	0 ± 0

Figure 9: Non-specific binding of IgM antibodies in the ELISPOT assay. (a) Representative wells display IgG and IgM spots detected in the wells coated with GPIIbIIIa, GPIbIX, anti-human IgG, anti-human IgM antibodies, wells without coating (no coat) and wells without PBMCs incubation. 300 000 PBMCs were incubated in the antigen-coated wells and 10 000 PBMCs were incubated in total IgG, total IgM, and no coat wells. (b) Representative wells display IgG, IgA and IgM spots detected in the wells with various coating conditions. Results are indicated as mean ± 1 SD of 4 replicates.

a)



b)



c)

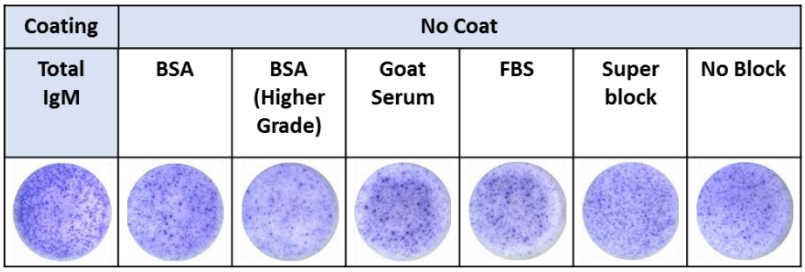


Figure 10: Optimizing the ELISPOT assay to minimize non-specific binding of IgM antibodies. 10 000 PBMCs were added to anti-human IgM coated wells and wells without coating antibodies (no coat). Spots were detected with biotinylated anti-human IgM antibodies. Factors tested include (a) binding capacity of the ELISPOT plate membrane, (b) cell incubation time and (c) types of blocking buffers. Each factor was tested using stimulated PBMCs from different donors. Representative wells were used as qualitative analysis for the presence or absence of non-specific binding of IgM spots on the no coat wells.

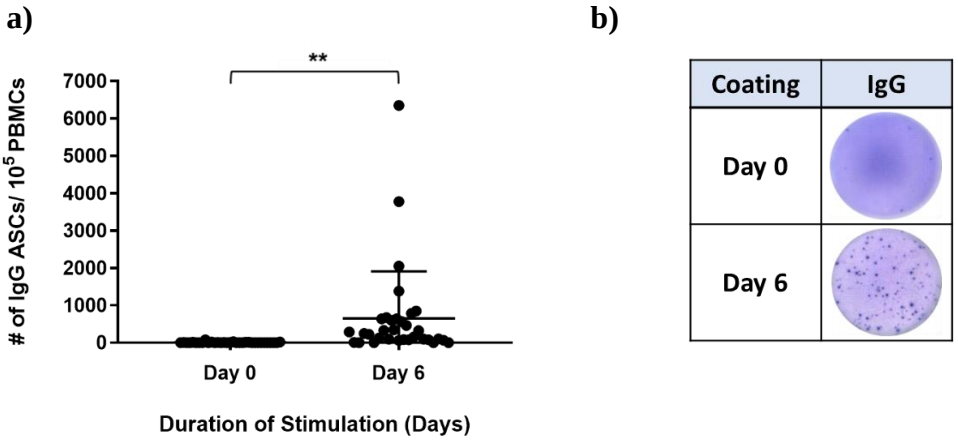


Figure 11: Total IgG ASCs in unstimulated and day 6 stimulated PBMCs. (a) The number of total IgG ACSs were detected before stimulation and after day 6 of stimulation (n=33). Results are expressed as the number of spots detected in 100 000 PBMCs with mean ± 1 SD. A paired t-test was used to determine the significance of the number of spots detected before and after stimulation of PBMCs (p=0.006). (b) Representative wells display total IgG spots before and after day 6 of stimulation.

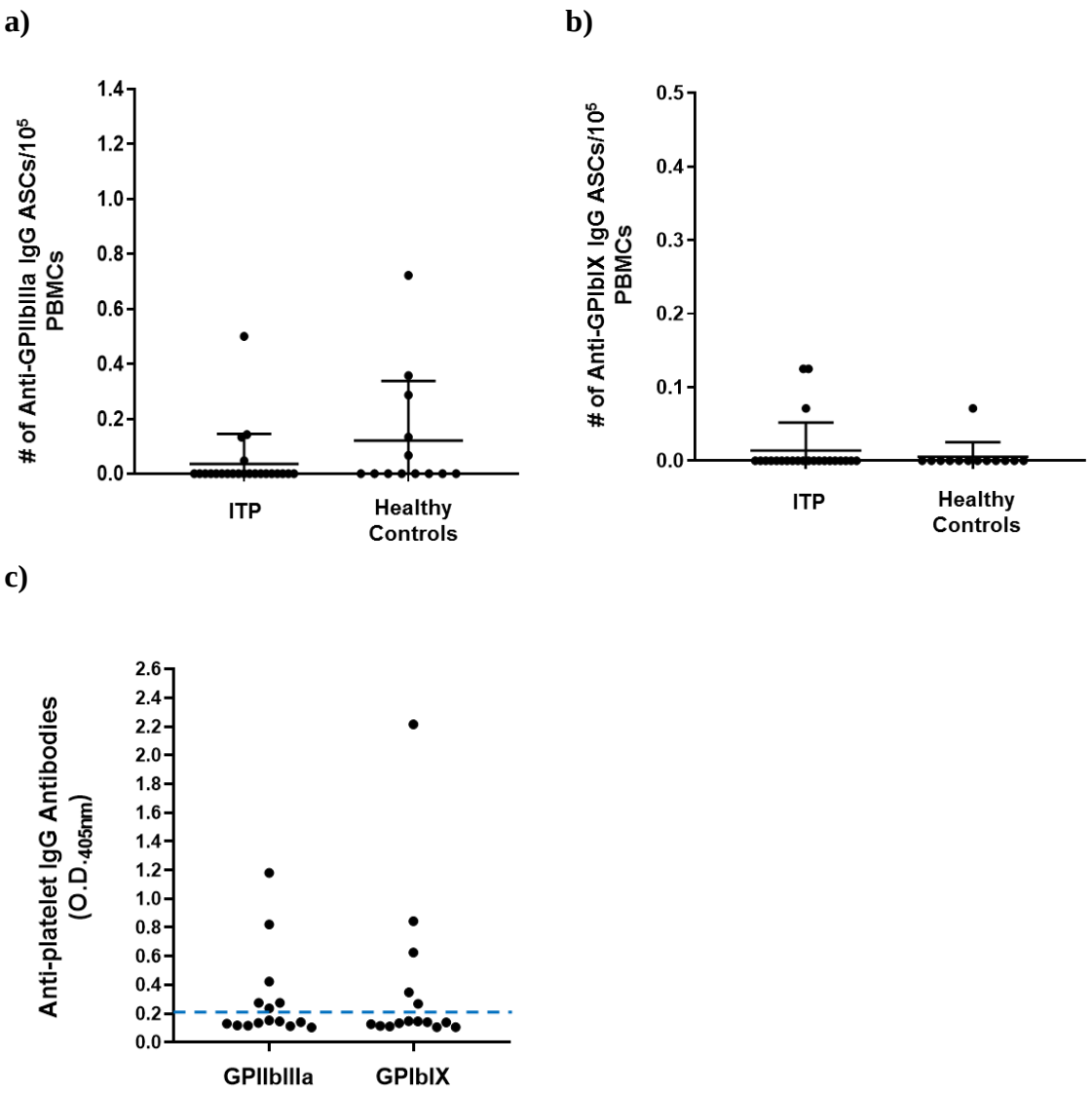


Figure 12: Antigen-specific IgG autoantibodies and ASCs in the peripheral blood of ITP patients. PBMCs were tested for the presence of (a) anti-GPIIbIIIa and (b) anti-GPIbIX IgG ASCs on day 6 of stimulation (ITP n=23, healthy controls n=13). Results are expressed as mean ± 1 SD. An unpaired t-test was used to determine the significance of the number of spots detected in ITP patients and healthy controls (GPIIbIIIa p=0.127 and GPIbIX p=0.461). (c) Anti-GPIIbIIIa and anti-GPIbIX IgG autoantibodies were detected using the direct antigen capture assay (n=15 ITP). The pre-established cut-off for positivity is indicated by the blue dotted line at O.D. of 0.21.

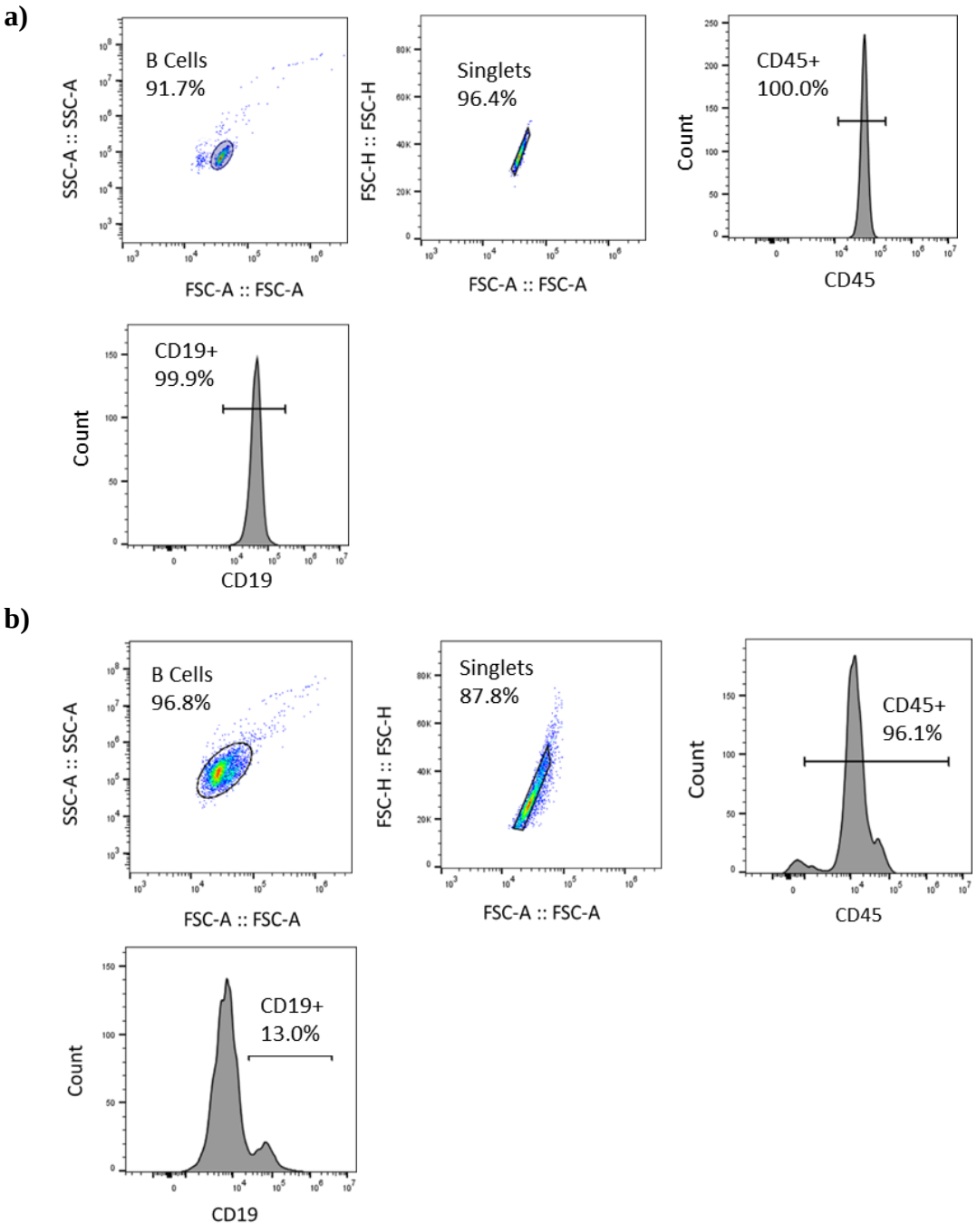


Figure 13: Proportion of CD19+ B cells before and after stimulation of purified B cells. (a) B cells were purified from PBMCs, and the purity was determined using anti-CD45 and anti-CD19 markers. (b) The proportion of CD19+ B cells were also identified after day 6 of stimulating purified B cells with R848 and IL-2.

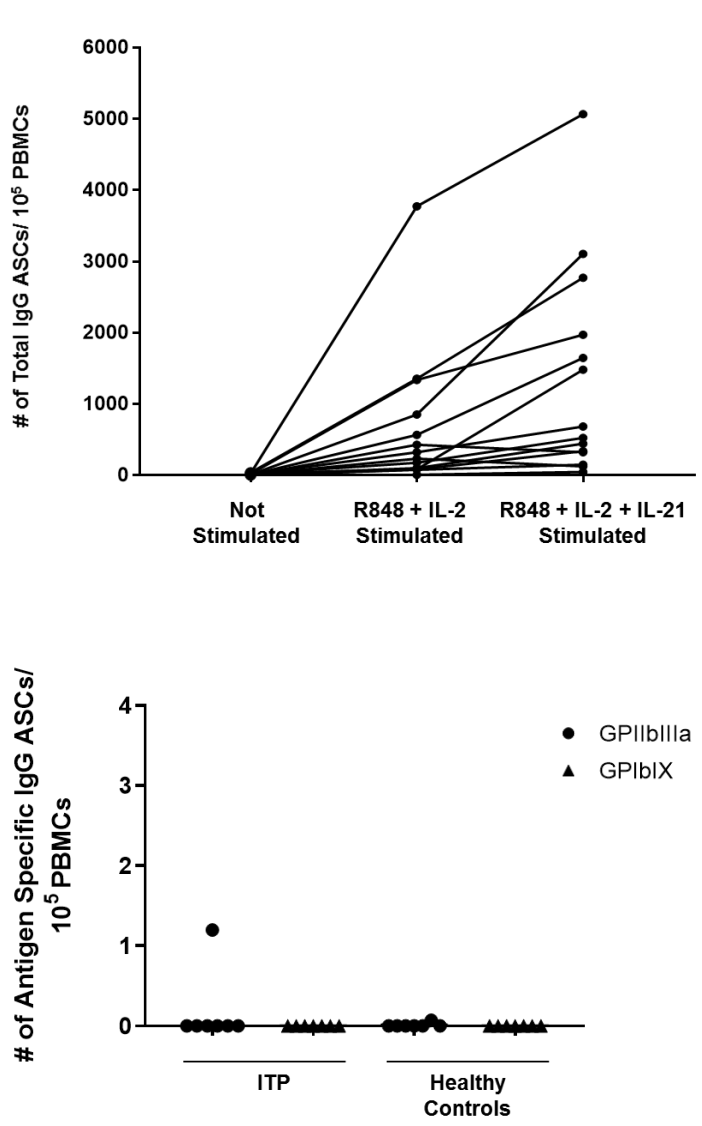


Figure 14: Detection of antigen-specific and total IgG ASCs in PBMCs stimulated with R848, IL-2, and IL-21. (a) Comparison of IgG ASCs detected before PBMCs stimulation, after stimulation with R848 and IL-2 and after stimulation with R848, IL-2, and IL-21 (n=14). (b) PBMCs stimulated with R848, IL-2, and IL-21 were tested for the presence of anti-GPIIbIIIa and anti-GPIbIX IgG ASCs (ITP patients n=7, healthy controls n=7).

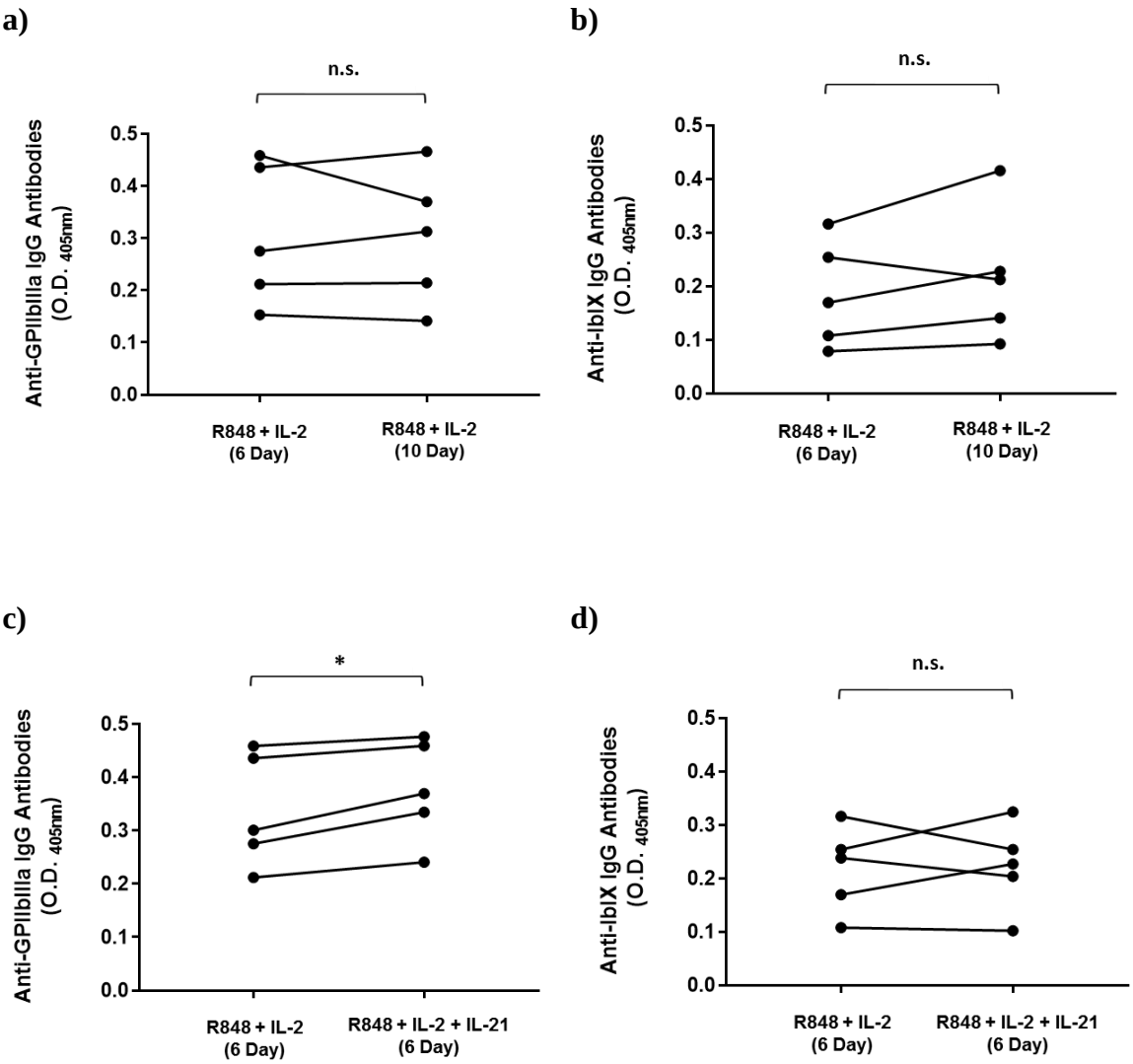


Figure 15: Detection of antigen-specific IgG autoantibodies in the supernatants of PBMCs. Supernatants from PBMCs were tested for the presence of autoantibodies using the indirect ELISA. (a,b) Anti-GPIIbIIIa and anti-GPIbIX IgG autoantibodies were compared in the supernatants of PBMCs stimulated with R848 and IL-2 for 6 days to 10 days (ITP n=5). A paired t-test indicated no significant difference in the presence of anti-GPIIbIIIa (p=0.806) and anti-GPIbIX autoantibodies (p=0.236). (c,d) Anti-GPIIbIIIa and anti-GPIbIX IgG autoantibodies were compared in the supernatants of PBMCs stimulated with R848 and IL-2 for 6 days to PBMCs stimulated with R848, IL-2, and IL-21 for 6 days (n=5). A paired t-test indicated a significant increase in the presence of anti-GPIIbIIIa autoantibodies (p=0.019) and no significant difference in the presence of anti-GPIbIX autoantibodies (p=0.850).

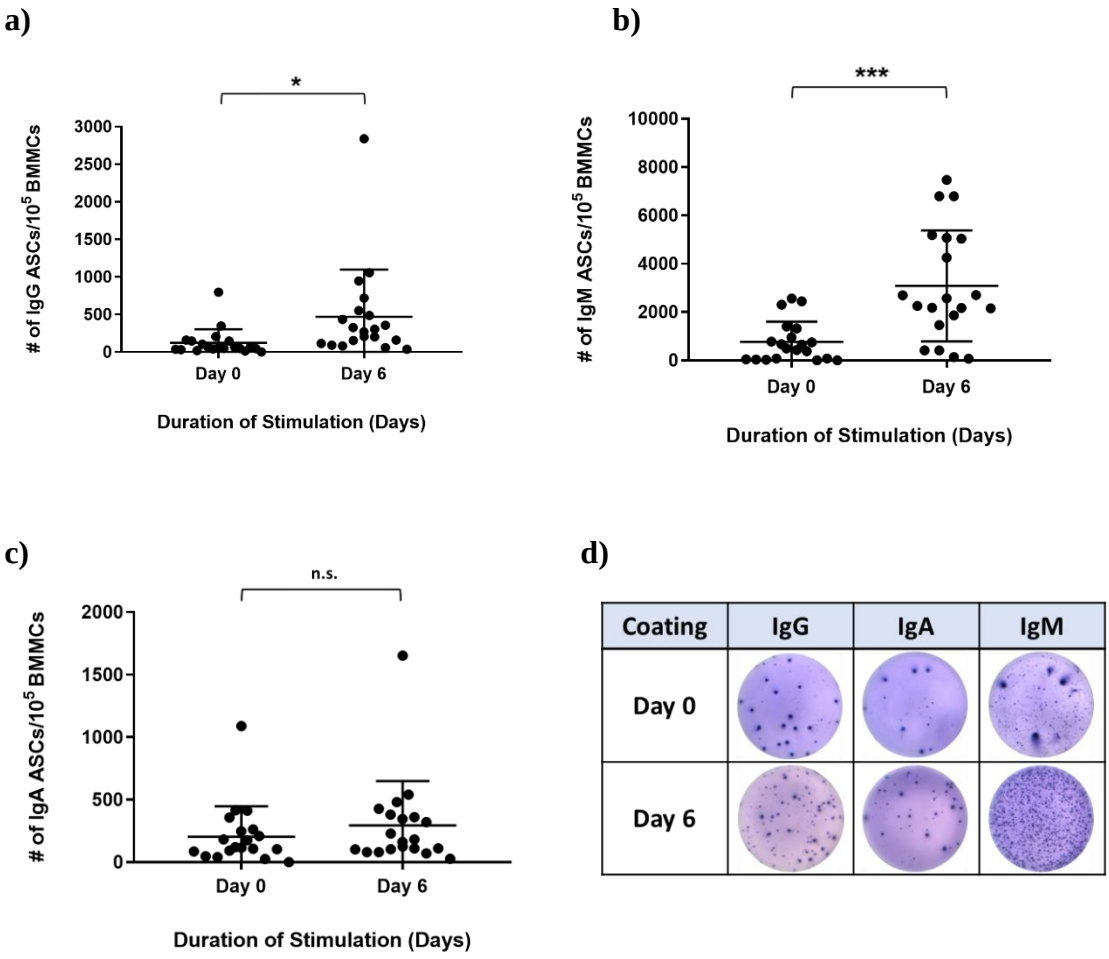
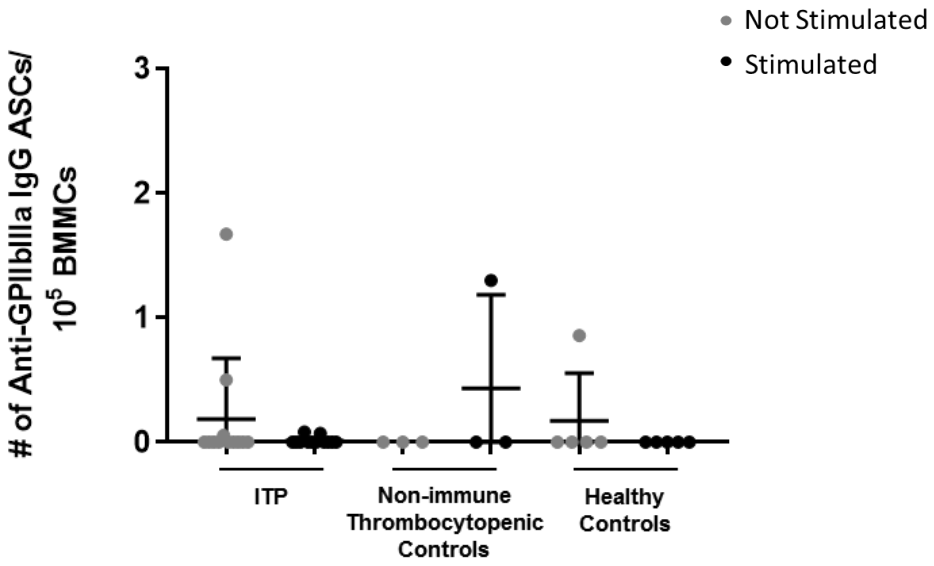
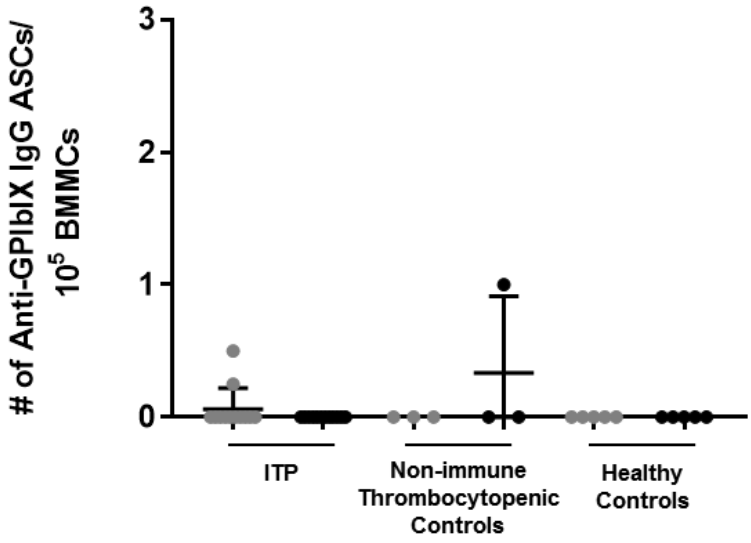


Figure 16: Detection of total IgG, IgA, and IgM ASCs in unstimulated and stimulated BMMCs. Total IgG, IgA, and IgM ASCs were detected in BMMCs before stimulation and on day 6 of stimulation with R848 and IL-2 (n=20). A paired t-test was used to determine the significance of the number of total IgG ASCs (p=0.027), total IgM ASCs (p=0.0003) and total IgA ASCs (p=0.324) detected in the unstimulated and stimulated conditions. Results are also expressed as mean ± 1 SD. (d) Representative wells display total IgG, IgA and IgM spots detected in 10 000 BMMCs before stimulation and after day 6 of stimulation.

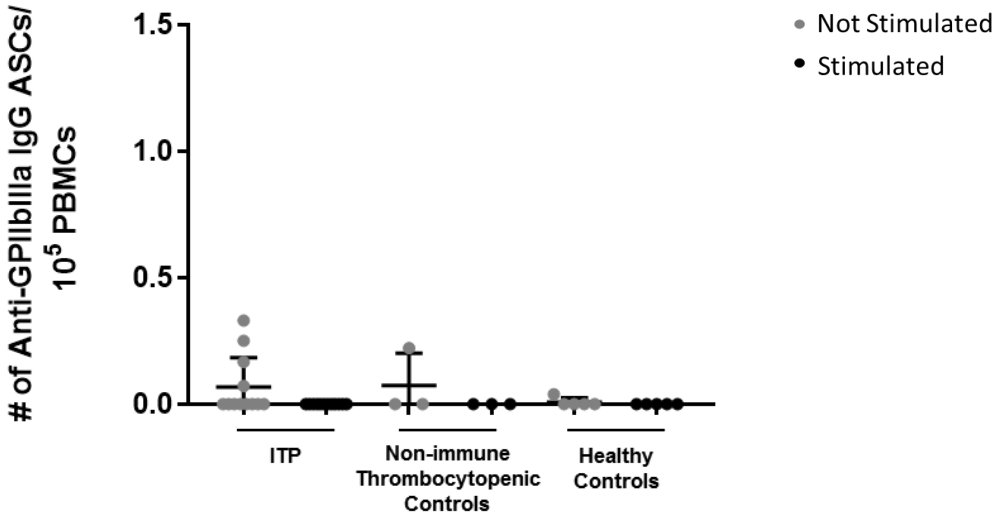
a)


Not Stimulated
Stimulated
# of Anti-GPIIbIIIa IgG ASCs/ 10^5 BMMCs
3
2
1
0
ITP
Non-immune Thrombocytopenic Controls
Healthy Controls

b)


# of Anti-GPIbIX IgG ASCs/ 10^5 BMMCs
3
2
1
0
ITP
Non-immune Thrombocytopenic Controls
Healthy Controls

c)



d)

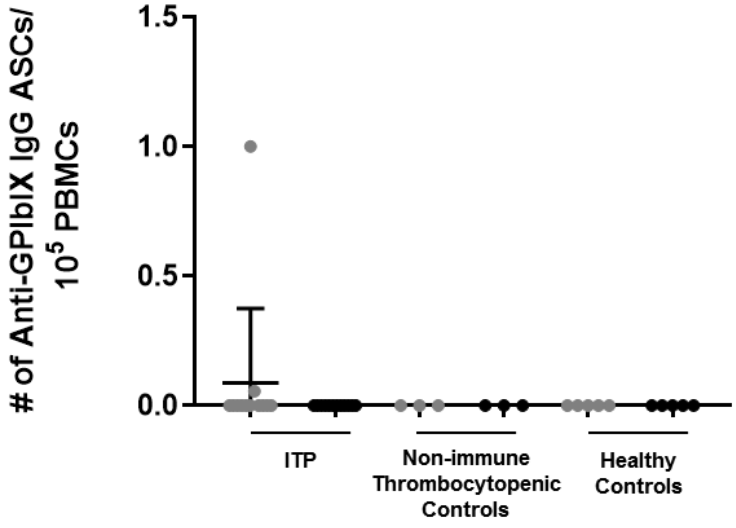
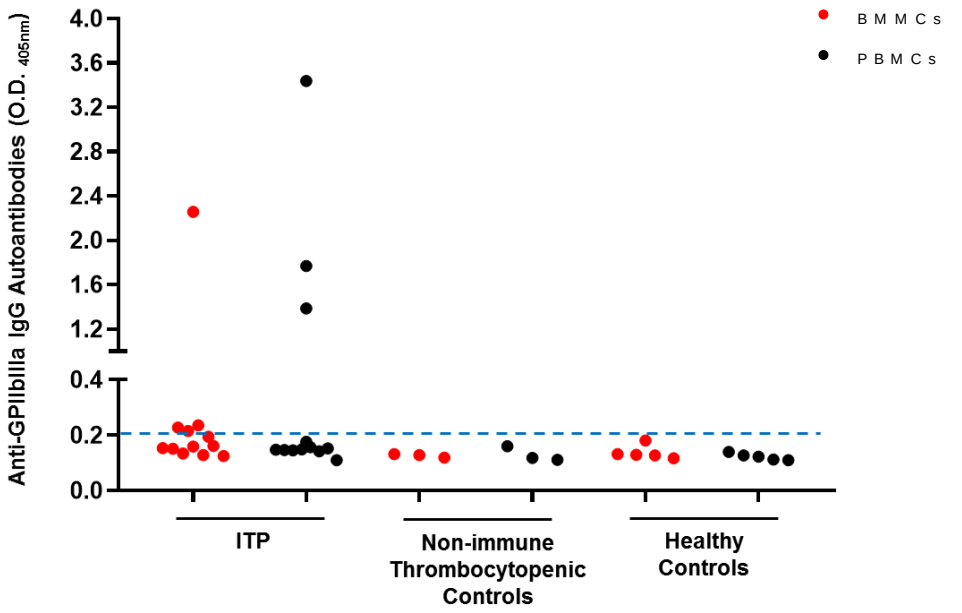


Figure 17: Quantifying antigen-specific IgG ASCs in the bone marrow and the peripheral blood of ITP patients and controls. The presence of antigen-specific ASCs in ITP patients (n=12), non-immune thrombocytopenic patient controls (n=3) and healthy controls (n=5) were tested using the ELISPOT assay. Unstimulated and stimulated BMMCs were tested for (a) anti-GPIIbIIIa and (b) anti-GPIbIX ASCs. Unstimulated and stimulated PBMCs were tested for (c) anti-GPIIbIIIa and (d) anti-GPIbIX ASCs.

a)



b)

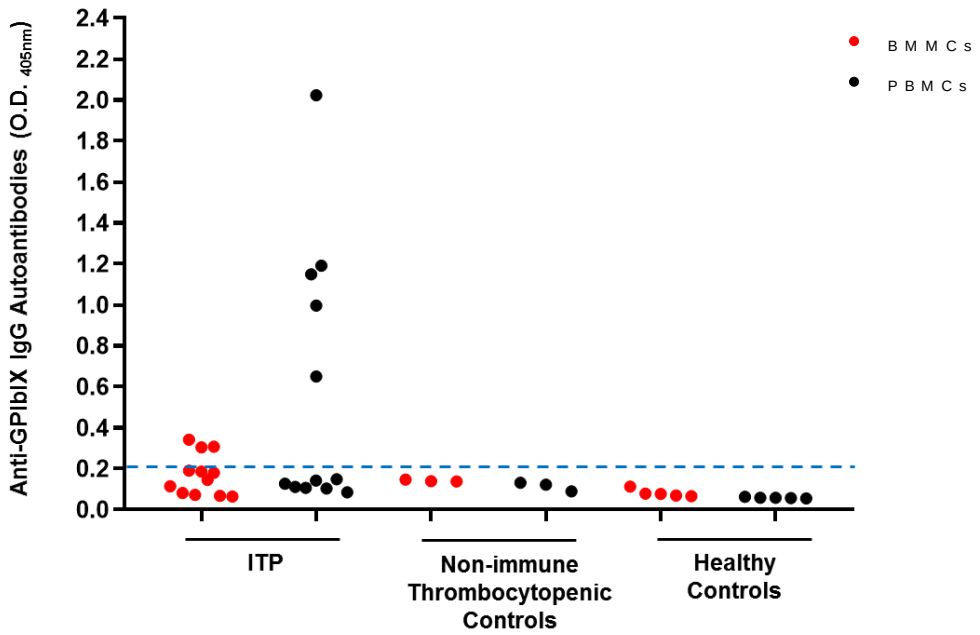


Figure 18: Antigen-specific IgG autoantibodies in the bone marrow and the peripheral blood of ITP patients and controls. The presence of antigen bound IgG autoantibodies were detected in ITP patients (n=12), non-immune thrombocytopenic patient controls (n=3) and healthy controls (n=5) using the direct AC assay. Bone marrow and peripheral blood samples were tested for the presence of (a) anti-GPIIbIIIa and (b) anti-GPIbIX autoantibodies. The pre-established cut-off is indicated by the blue dotted line at an O.D. of 0.21.

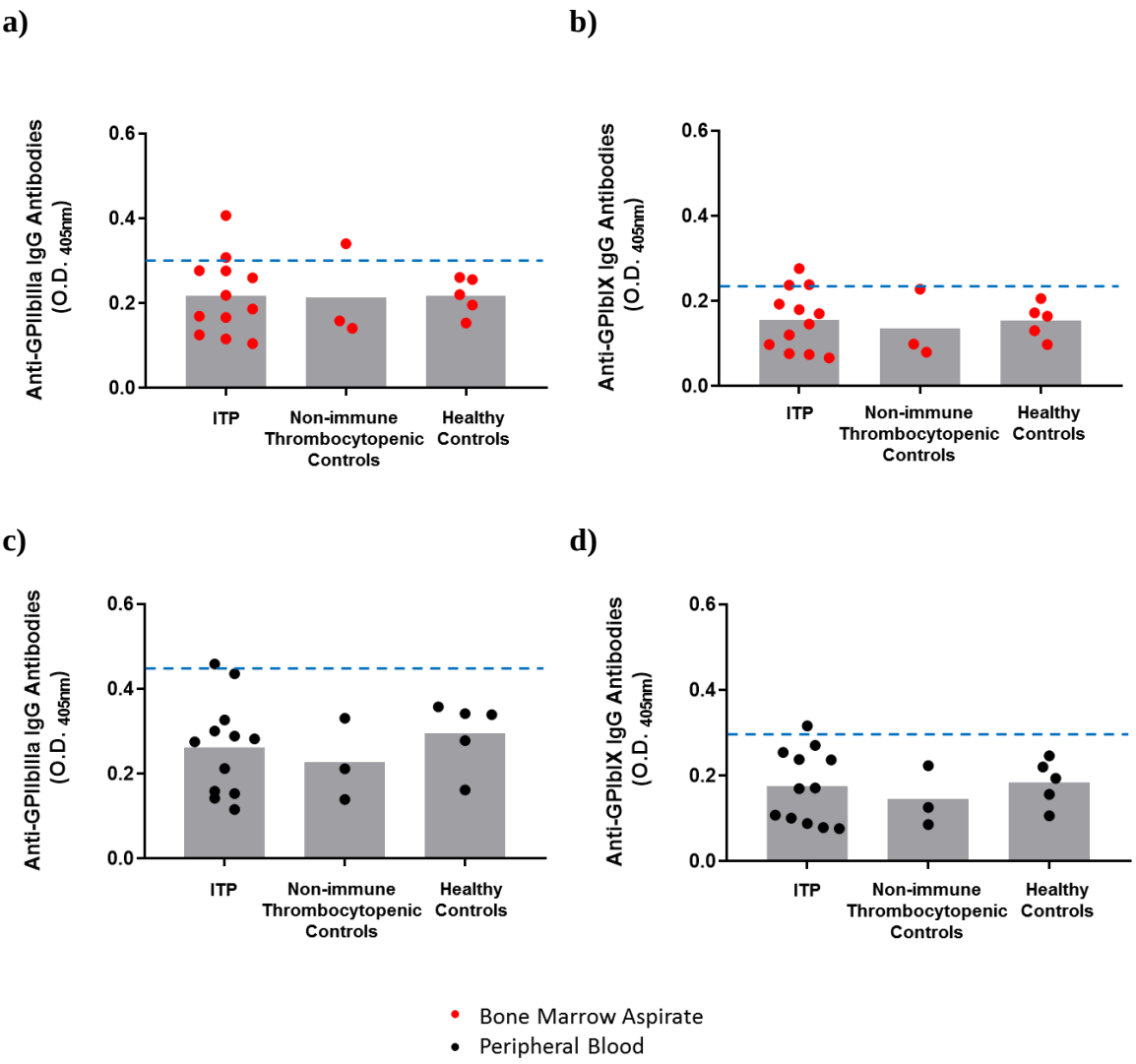
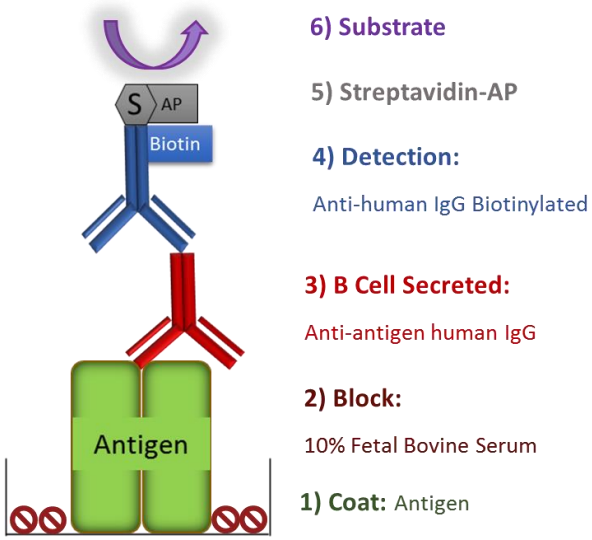


Figure 19: Detection of antigen-specific IgG autoantibodies in the supernatants of BMMCs and PBMCs cultures. BMMCs and PBMCs from ITP patients (n=12), non-immune thrombocytopenic patient controls (n=3), and healthy controls (n=5) were cultured in media during stimulation with R848 and IL-2 for 6 days. The BMMCs supernatants were used in the indirect ELISA to detect (a) anti-GPIIbIIIa (cut-off 0.31) and (b) anti-GPIbIX (cut-off 0.24) IgG antibodies. The PBMCs supernatants were used in the indirect ELISA to detect (c) anti-GPIIbIIIa (cut-off 0.46) and (d) anti-GPIbIX (cut-off 0.29) IgG antibodies. The cut-off values were determined as 2 SD above the mean of healthy controls for each type of mononuclear cells and the specificity for each antigen as indicated by the blue dotted line. The bar represents the mean O.D. for the ITP patient and the control groups.

a)



b)

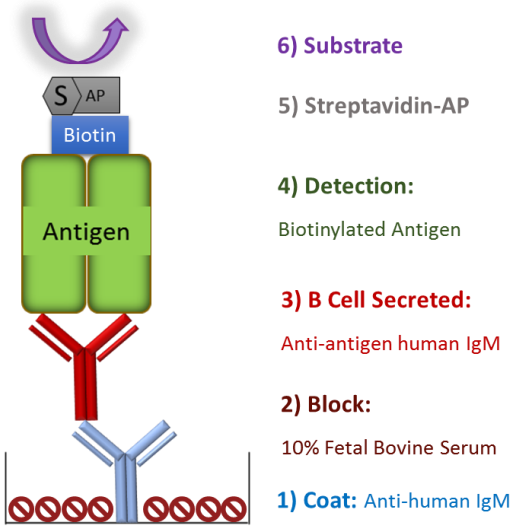


Figure 20: Schematic diagram of variations of the ELISPOT assay to detect antigen-specific IgG, IgA, and IgM ASCs. (a) Antigen-specific IgG and IgA ASCs can be detected by coating the ELISPOT plate wells with the antigen to capture antigen-specific IgG, and IgA antibodies secreted by ASCs and are detected using biotinylated anti-human IgG and IgA antibodies, respectively. (b) Antigen-specific IgM ASCs can be detected by coating the ELISPOT plate wells with anti-human IgM antibodies to capture IgM antibodies secreted by ASCs and is detected with biotinylated antigen.

9. TABLES:

Table 1. Total IgG ASCs detected in isolated B Cells and PBMCs.

Experiment	**B cell Isolation from PBMCs** (% of PBMCs)	**B cell isolation & stimulation** (# of IgG ASCs/ 10^4 B Cells)	**PBMCs Stimulation & B cell isolation** (# of IgG ASCs/ 10^4 B Cells)	**PBMCs stimulation:** (# of IgG ASCs/ 10^4 PBMCs)
1	11.85%	14 ± 2	N/A	25 ± 6
2	2.21%	2 ± 0	30 ± 3	17 ± 2

Table 2. Autoantibodies and autoreactive B cells in the bone marrow and the peripheral blood of ITP patients

ITP Patients Chronic	Platelet Count (x10^9/L)	Antigen Specificity	Bone Marrow			Peripheral Blood		
			ELISPOT Assay (/10^5 PBMCs)		AC Assay (O.D.)	ELISPOT Assay (/10^5 PBMCs)		AC Assay (O.D.)
			Day 0	Day 6		Day 0	Day 6	
1	29	GPIIbIIIa	**0.06**	0.00	**0.236**	0.00	0.00	**1.770**
		GPIbIX	0.00	0.00	**0.308**	0.00	0.00	**1.149**
2	34	GPIIbIIIa	0.00	**0.08**	0.195	0.00	0.00	0.110
		GPIbIX	0.00	0.00	**0.342**	0.00	0.00	**2.024**
3	34	GPIIbIIIa	0.00	**0.07**	**0.215**	**0.07**	0.00	0.149
		GPIbIX	0.00	0.00	0.114	0.00	0.00	0.112
4	13	GPIIbIIIa	0.00	0.00	0.159	**0.25**	0.00	0.153
		GPIbIX	0.00	0.00	0.082	0.00	0.00	0.085
5	20	GPIIbIIIa	**0.50**	0.00	0.154	**0.17**	0.00	0.177
		GPIbIX	**0.25**	0.00	0.180	0.00	0.00	0.149
6	3	GPIIbIIIa	**1.67**	0.00	0.162	**0.33**	0.00	**1.387**
		GPIbIX	**0.50**	0.00	0.191	**1.00**	0.00	**0.650**
7	53	GPIIbIIIa	0.00	0.00	0.134	0.00	0.00	0.157
		GPIbIX	0.00	0.00	0.146	0.00	0.00	0.142
8	52	GPIIbIIIa	0.00	0.00	0.129	0.00	0.00	0.146
		GPIbIX	0.00	0.00	0.072	0.00	0.00	0.103
9	99	GPIIbIIIa	0.00	0.00	**2.258**	0.00	0.00	**3.439**
		GPIbIX	0.00	0.00	0.065	0.00	0.00	**0.996**
10	84	GPIIbIIIa	0.00	0.00	0.151	0.00	0.00	0.147
		GPIbIX	0.00	0.00	0.187	**0.06**	0.00	**1.191**
11	49	GPIIbIIIa	0.00	0.00	**0.228**	0.00	0.00	0.142
		GPIbIX	0.00	0.00	**0.304**	0.00	0.00	0.127
12	35	GPIIbIIIa	0.00	0.00	0.125	0.00	0.00	0.148
		GPIbIX	0.00	0.00	0.068	0.00	0.00	0.106

Table 3. Autoantibodies and autoreactive B cells in the bone marrow and the peripheral blood of non-immune thrombocytopenic patient controls

Non-immune Thrombocytopenic Patients	Platelet Count (x10^9/L)	Antigen Specificity	Bone Marrow			Peripheral Blood		
			ELISPOT Assay (/10^5 PBMCs)		AC Assay (O.D.)	ELISPOT Assay (/10^5 PBMCs)		AC Assay (O.D.)
			Day 0	Day 6		Day 0	Day 6	
1 (Progressive pancytopenia)	95	GPIIbIIIa	0.00	0.00	0.119	0.00	0.00	0.118
		GPIbIX	0.00	0.00	0.140	0.00	0.00	0.123
2 (Fanconi's anemia)	39	GPIIbIIIa	0.00	**1.30**	0.132	**0.22**	0.00	0.160
		GPIbIX	0.00	**1.00**	0.147	0.00	0.00	0.132
3 (Splenomegaly)	59	GPIIbIIIa	0.00	0.00	0.129	0.00	0.00	0.111
		GPIbIX	0.00	0.00	0.138	0.00	0.00	0.090

Table 4. Autoantibodies and autoreactive B cells in the bone marrow and the peripheral blood of healthy controls

Healthy Controls	Platelet Count (x10^9/L)	Antigen Specificity	Bone Marrow			Peripheral Blood		
			ELISPOT Assay (/10^5 PBMCs)		AC Assay (O.D.)	ELISPOT Assay (/10^5 PBMCs)		AC Assay (O.D.)
			Day 0	Day 6		Day 0	Day 6	
1	100	IIbIIIa	0.00	0.00	0.181	0.00	0.00	0.140
		IbIX	0.00	0.00	0.113	0.00	0.00	0.063
2	330	IIbIIIa	0.00	0.00	0.132	0.00	0.00	0.110
		IbIX	0.00	0.00	0.079	0.00	0.00	0.058
3	115	IIbIIIa	**0.86**	0.00	0.127	**0.04**	0.00	0.127
		IbIX	0.00	0.00	0.077	0.00	0.00	0.059
4	100	IIbIIIa	0.00	0.00	0.117	0.00	0.00	0.112
		IbIX	0.00	0.00	0.067	0.00	0.00	0.057
5	190	IIbIIIa	0.00	0.00	0.130	0.00	0.00	0.123
		IbIX	0.00	0.00	0.070	0.00	0.00	0.056

Table 5. Autoantibodies in the bone marrow and the peripheral blood compartments of ITP patients

Chronic ITP	Splenectomy	Bone Marrow GPIIbIIIa	Bone Marrow GPIbIX	Peripheral Blood GPIIbIIIa	Peripheral Blood GPIbIX
		Autoantibodies (cut-off O.D. 0.21)			
1	No	+	+	+	+
2	No	-	+	-	+
3	No	+	-	-	-
4	No	-	-	-	-
5	N/A	-	-	-	-
6	N/A	-	-	+	+
7	N/A	-	-	-	-
8	N/A	-	-	-	-
9	Yes	+	-	+	+
10	Yes	-	-	-	+
11	Yes	+	+	-	-
12	Yes	-	-	-	-

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