ANTIPLATELET ANTIBODY INHIBITION OF PLATELET PRODUCTION

INVESTIGATING AUTOANTIBODIES IN THE PATHOPHYSIOLOGY OF PLATELET UNDERPRODUCTION IN IMMUNE THROMBOCYTOPENIA

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TITLE: Investigating Autoantibodies in the Pathophysiology of Platelet Underproduction in Immune Thrombocytopenia

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Lay Abstract

Immune thrombocytopenia (ITP) is an immune mediated blood disorder where autoantibodies target and destroy platelets, cells that are crucial for preventing blood loss. Evidence from different studies show that ITP autoantibodies are also affecting the cells producing platelets (megakaryocytes), but the mechanism of this effect remains unknown. To study antibody mediated inhibition of megakaryopoiesis, I developed a peripheral blood based megakaryopoiesis assay that used the patient's own cells as a starting source to grow megakaryocytes. With this assay, I investigated the effect patient plasma had on platelet production. I found no inhibition of megakaryocyte growth but did find an effect on their ability to produce platelets. I also found a model antibody that affected the maturity of the megakaryocytes during their development. These tools can now be used to further investigate impaired platelet production in ITP patients and determine the impact this inhibition has on ITP pathology.

Abstract

Immune thrombocytopenia (ITP) is a heterogeneous immune-mediated blood disorder with multiple pathologies that cause thrombocytopenia. The primary source of this thrombocytopenia is platelet destruction by antiplatelet autoantibodies. Although several treatment options are available for ITP, they are often transient, and responses can be difficult to predict. Different studies show ITP plasma and autoantibodies can also inhibit platelet production, but the mechanism and its impact in causing thrombocytopenia remains unknown. By identifying the different mechanisms causing

ITP thrombocytopenia, it may be possible to identify more effective and patient specific treatment options, as well as identify patients who could be at an increased risk of bleeding. To study the antibody mediated inhibition of platelet production, I developed a peripheral blood based megakaryopoiesis assay that used the patient's own hematopoietic stem and progenitor cells (HSPC) as a starting cell source to grow megakaryocytes. I demonstrate this assay can use a small amount of peripheral blood to grow mature megakaryocytes that are capable of thrombopoiesis. Using this assay, I investigated the effect patient plasma had on platelet production. As such, this study is the first autologous investigation of the effect ITP plasma has on platelet production. I found no inhibition of megakaryopoiesis, but did find an effect on thrombopoiesis, indicating that the plasma is affecting the end stages of platelet production. Secondary observations also show that some ITP HSPC have an enhanced megakaryopoiesis potential, generating more mature megakaryocytes than what was observed with healthy donors. While screening monoclonal antiplatelet antibodies, I discovered an anti-GPIb antibody that inhibited megakaryocyte maturation and found this affect was also present with the Fab antibody fragment. From my research I have developed several tools that can be used to investigate impaired platelet production in ITP and further our understanding of this pathology.

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giving up and how to be aggressive when it comes to research and academia, and to always aim high. I would also like to thank Boki Misic, Marcia Reid and Reza Jefari for their technical support.

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List of all Abbreviations and Symbols

(7AAD)	7-Aminoactinomycin D
(ACD)	Acid citrate dextrose
(ACA)	Antigen capture assays
(AFT)	Albumin Free Tyrode's buffer
(AMR)	Ashwell Morell receptor
(APC)	Allophycocyanin
(BFU)	Burst Forming Units
(BSA)	Bovine serum albumin
(CAF)	Calcium/albumin-free Tyrode's solution
(CD)	Clusters of differentiation
(CFU-MK)	Colony forming units-megakaryocytes
(CMP)	Common myeloid progenitors
(DAPI)	4′,6-diamidino-2-phenylindole
(DMS)	Demarcated membrane system
(DTS)	Dense tubular system
(EIA)	Enzyme linked immunosorbent assay
(EtOH)	Ethanol
(EDTA)	Ethylenediaminetetraacetic
(FITC)	Fluorescein isothiocyanate
(GPIb or CD42b)	Glycoprotein Ib
(GPIIb/IIIa, CD41/61, CD41a)	Glycoprotein IIb/IIIa
(HSPCs)	Hematopoietic stem and progenitor cells
(HSC)	Hematopoietic stem cells
(HLA)	Human leukocyte antigens
(ITP)	Immune thrombocytopenia
(IgG)	Immunoglobulin G
(IVIG)	Intravenous immunoglobulin
(kDa)	Kilodaltons
(MEP)	Megakaryocyte-erythrocyte progenitors
(OCS)	Open canalicular system
(OD280)	Optical density 280
(PBMCs)	Peripheral blood monocular cells
(PBS)	Phosphate-buffered saline
(PE)	Phycoerythrin
(PECy5)	Phycoerythrin-cyano 5
(PECAM-1)	Platelet endothelial cell adhesion molecule
(PF4)	Platelet factor 4

(RES)	Reticuloendothelial system
(SRA)	Serotonin release assay
(SDS-PAGE)	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
(SCF)	Stem cell factor
(SDF-1)	Stromal development factor-1.
(TPO)	Thrombopoietin
(VWF)	Von-Willebrand factor

Declaration of Academic Achievements

The experiments outlined in this thesis were designed, conducted, and interpreted by myself and my supervisors, Dr. Ishac Nazy, Dr. Donald M. Arnold, and Dr. John G. Kelton, unless specified otherwise in the author preface. All SDS-PAGE were done by James Smith or Dr. Angela H. Huynh (Chapter 3). All SRAs were performed by Hina Bhakta and James W. Smith (Chapter 3). DLS experiments were performed by Dr. Angela H. Huynh. Marcia Reid from the Faculty of Health Science's Electron Microscopy Facility helped develop the processed samples for electron microscopy imaging (Chapter 2).

Chapter 1

Introduction

1.1 Platelets

Platelets (thrombocytes) are blood cells crucial for the formation of clots, which prevent bleeding.[1] These cells circulate in blood at concentrations of 150-350 $*10^9$/litre constantly screening blood vessels for signs of injury.[1,2] After binding to exposed subendothelial proteins or agonists, platelets become activated.[1,3] Platelet activation is characterized by several rapid functional and morphological changes. The platelet restructures itself, expanding in size and altering its membrane to become a catalytic surface for coagulation proteins.[1] It secretes granules into the local environment that support coagulation and stimulate other nearby platelets to activate.[4,5] Activated platelets become adherent, aggregating to form a platelet plug to block the damaged vessel and prevent additional blood loss; this process is termed primary hemostasis.[6] Platelets are

also involved in tissue repair, angiogenesis and help support the immune system against invading pathogens.[7,8] Thrombocytopenia is a blood condition defined by low levels of circulating platelets and this increases the risk and severity of bleeding. [9]

1.2 Platelet Physiology

Platelet's unique morphology supports its function in maintaining hemostasis. They have a discoid shape and are significantly smaller than other blood cells with an average diameter of 2-5 microns.[1] This small size causes the cells to be pushed towards the edges of the blood vessel under flow conditions, resulting in an increase in interactions with the vascular structure.[11] The platelet's discoid shape is maintained by an internal microtubule bundle of rings supported by an actin and sceptrin cytoskeleton.[1,12] Upon activation, the cytoskeleton is reorganized, the platelet extends pseudopods outwards from the cell to help form attachments and begins to flatten its shape.[1,13]

Platelets possess two different internal membrane systems: the dense tubular system (DTS) and the open canalicular system (OCS).[1] The DTS is a series of close channels permeating throughout the cell which function as sites of prostaglandin synthesis and calcium storage.[14] The OCS is a series of open-ended channels that invaginate throughout the cell and are continuous with the cell membrane. The OCS is believed to act as a membrane reserve that is released upon activation, supporting the formation of the platelet pseudopods and increasing the cell's surface area over 400%.[15]

The expanded membrane is the centre for secretion of platelet specific granules whose contents aid in platelet adhesion, activation, and support of the coagulation cascade.[5,15]

Upon activation, platelets release dense (δ) and α-granules to aid in forming a platelet plug.[1] These dense δ granules (3-8 per cell) contain ions such as calcium and magnesium along with signalling molecules such as serotonin, histamine, and ADP.[4,16] These ions and molecules have various effects on numerous cells, including stimulating nearby platelets to activate and acting as vasoconstrictors to restrict blood flow to the damaged blood vessel.[4] The α-granules (50-80 per cell) in each platelet contain various proteins such as platelet factor 4 (PF4), fibrinogen, and von-Willebrand factor (VWF) amongst others as well as platelet receptors like P-selectin and PECAM.[4,5] These proteins aid in platelet activation and adhesion, support the coagulation cascade and function as cytokines and growth factors for nearby cells.[4,8]

Platelets do not possess a nucleus and thus do not undergo mitosis or maintain protein production for an extended length of time.[1,17] The platelet lifespan is approximately 10 days after which time the cell undergoes apoptosis and clearance by the reticuloendothelial system (RES).[17] To maintain normal platelet levels, humans produce over $1.0*10^8$ platelets daily, and this is accomplished in the bone marrow by cells called megakaryocytes.[2]

1.3 Megakaryocytes

Megakaryocytes are progenitor cells that produce platelets. A single mature megakaryocyte is estimated to produce 2,000-5,000 platelets during its lifespan.[18,19] Megakaryocytes reside in the bone marrow and comprise approximately 0.01% of the total cell population.[20] Megakaryocytes have evolved a unique physiology and morphology that aids in their ability to produce platelets. Mature megakaryocytes are large cells with a measured diameter of up to 100 microns in length.[21] They are polyploid and contain multiple chromosome copies with measured DNA levels ranging from 4 n to 128 n whereas a typical cell is diploid and contains only two chromosome copies (2n).[21] Evidence suggests this large amount of DNA is necessary to sustain a high level of protein production as the megakaryocyte produces the platelet receptors, proteins, and granules required for a platelet to function.[18] Megakaryocytes possess a demarcated membrane system (DMS) consisting of invaginated membrane channels permeating throughout the cell.[22] This membrane may act as a reservoir the megakaryocyte uses to produce platelets by a process called thrombopoiesis. [23]

1.4 Thrombopoiesis

Mature megakaryocytes produce platelets by undergoing a process known as thrombopoiesis.[23] As a megakaryocyte matures it migrates towards blood vessels located in the bone marrow, moving from the endosteal niche to the sinusoidal vascular niche.[24]

This migration is supported by concentration dependent chemotactic signalling, most notably by stromal development factor-1 (SDF-1).[24] Upon reaching a blood vessel, the megakaryocyte receives stimulation by various matrix proteins to initiate thrombopoiesis.[25,26] This process begins with the altering of the megakaryocyte cytoskeleton to produce long proplatelet extensions from its body. These extensions penetrate the blood vessel, and under the sheer force from the blood flow, they break off and develop into platelets.[27] Detailed microscopy demonstrates that the tips of the proplatelet extensions resemble the microtubule bundles found in platelets suggesting these are the direct sites of platelet production.[28] Whole megakaryocytes can also enter the blood vessel where they are caught in pulmonary capillaries and shredded into platelets.[29] Certain cytokines, such as IL-1α, have also been shown to initiate a rapid form of thrombopoiesis and megakaryocyte fragmentation that could be a method for immediate platelet production.[30] Megakaryocytes perform thrombopoiesis for several hours until the process consumes its entire membrane and cytoplasm, leaving behind a small nuclear husk that is then cleared.[26,27]

1.5 Megakaryopoiesis

The generation and maturation of megakaryocytes is called megakaryopoiesis. Since the process of thrombopoiesis results in the loss of intact megakaryocytes, new megakaryocytes must be continuously generated to maintain platelet production. [31] Like all blood cells, megakaryocytes originate from hematopoietic stem cells (HSC). HSC

undergo megakaryopoiesis following signalling by the cytokine thrombopoietin (TPO), the key regulator of platelet production.[32,33] After TPO stimulation, HSC progress through a series of progenitor stages with each stage representing a commitment to a certain cell lineage or subgroup.[34] These progenitors usually have lower proliferation rates and begin expressing lineage specific surface receptors.[34] These receptors are denoted as clusters of differentiation (CD) and indicate what kind of blood cells the progenitors are developing into.[34]

For megakaryopoiesis, the classic development pathway is HSC first committing to becoming common myeloid progenitors (CMP). These CMP progenitors then become megakaryocyte-erythrocyte progenitors (MEP) before becoming megakaryocyte only progenitors.[32,34] The earliest indicator a cell is undergoing megakaryopoiesis and will be a megakaryocyte is the expression of the platelet receptor glycoprotein (GP) IIb/IIIa (CD41/61 or CD41a).[35,36] Although this is the traditional megakaryocyte development pathway, new research suggests certain progenitor stages can be bypassed, perhaps representing an emergency mechanism of increasing platelet production, and so the current developmental hierarchy could be subject to change.[31,37] Once undergoing exclusively megakaryopoiesis, the megakaryocyte progenitors undergo maturation.

Megakaryocyte progenitors gradually lose the ability to proliferate before morphologically developing into mature megakaryocytes. The early progenitors are small $CD41a^+$ cells classified according to their rate of proliferation. BFU (Burst Forming

Units) megakaryocyte progenitors are capable of proliferating >100-fold before becoming CFU-MK cells (colony forming units) which can only proliferate <100 fold. [36,38] These early progenitors contain elevated RNA expression, prominent ribosomes, and rough endoplasmic reticulum. They have the highest known nuclear/cytoplasmic ratio of all the progenitors and have started developing platelet granules and the beginnings of a DMS.[38] Once the progenitor stops proliferating it undergoes endomitosis; a process in which the cell begins performing mitosis but aborts when reaching cytokinesis, resulting in a polyploid cell. This stop at cytokinesis is due to a loss of the contractile ring responsible for separating the two potential daughter cells and involves Rho/Rock signalling.[39] Endomitosis is repeated several times, resulting in the development of a polyploid cell. After this is completed, the cytoplasm rapidly expands as the cell begins maturing into a complete megakaryocyte. Platelet receptors such as CD41a and platelet organelles increase, while the DMS begins to expand.[35] The expression of glycoprotein Ib (CD42b) has been correlated with megakaryocyte polyploidy and is used as a marker for maturity.[40] Once mature, the megakaryocyte is ready to perform thrombopoiesis. This entire process is estimated to take five days following TPO stimulation in the bone marrow.[41]

1.6 Thrombopoietin and its regulation of platelet production

TPO is the key regulator of platelet production, responsible for inducing HSC into megakaryopoiesis.[42] TPO binds to the receptor C-MpL, a member of the JAK/STAT

receptor signalling family.[41] Once bound, TPO induces C-MpL to alter its dimerized shape and rotate its cytoplasmic tail, allowing bound JAK2 or TYK2 kinases to come into proximity and phosphorylate each other into activation.[43] These activated kinases then phosphorylate the cytoplasmic domain of C-Mpl which serves as a docking region for transcription factors such as STAT3 and STAT5 amongst other signalling proteins that then become active and cause downstream signal transduction.[43] Following activation, the TPO/C-Mpl complex is internalized and degraded, removing the bound TPO along with it.[44] High concentrations of TPO can induce megakaryopoiesis without additional cytokine signalling required but low concentrations are also known to support the survival of hematopoietic stem cells and early erythroid and myeloid progenitors.[45] Even though TPO initiates megakaryopoiesis, it is not necessary for the later stages of megakaryocyte maturation and thrombopoiesis.[46] Although TPO is not the only cytokine that can induce megakaryopoiesis, other cytokines such as GM-CSF, SCF, IL-3, IL-6, and IL-11 can in various combinations replicate its effect, removing the TPO gene results in a ~85% reduction in the number of circulating platelets in mouse models.[45,47,48]

TPO is produced at a constant rate in the liver and kidneys and is removed from circulation after binding to its target C-Mpl receptors, which are expressed on platelets, megakaryocytes and HSPC.[49,50] This establishes an auto-regulatory loop; if there is an adequate number of platelets then circulating TPO levels and stimulation for megakaryopoiesis would be low. If the number of platelets decreases, then TPO levels will rise and megakaryopoiesis and platelet production would be stimulated.[50] However,

this theory of regulation is recently being reviewed as new evidence suggests TPO production rates can be changed in hepatocytes via the Ashwell Morell receptor (AMR), which recognizes the glycosylation status of platelets. [51] As platelets age, they become desialylated and these desialylated platelets bind to the AMR receptors on hepatocytes where they are endocytosed and cleared from circulation. [51] This process stimulates the hepatocyte to increase TPO production. Investigating AMR's role in regulating platelet production is ongoing.[51]

1.7 Immune thrombocytopenia

Immune thrombocytopenia (ITP) is an acquired bleeding disorder identified by its clinical symptom of thrombocytopenia.[52] It has an autoimmune pathology where the immune system loses regulation and destroys the body's circulating platelets. [52] This causes thrombocytopenia which increases the risk and severity of bleeding. Bleeding symptoms can range from bruising and petechiae formation to rare but severe instances of intracranial hemorrhaging which can be fatal.[10,53] The estimated prevalence of ITP is 12.1 per 100,000 with an incidence rate of 3.3 per 100,000 in adults.[54] ITP is more common in women than men and can develop in children where a similar gender imbalance is present.[54]

ITP can be classified by its duration, association with other illnesses and treatment response. Patients can be defined as newly diagnosed, persistent (3-12 months) or chronic

ITP (>12 months) depending on the duration.[54,55] ITP usually resolves in most children but is often chronic in adults.[54] As a result, most ITP patients are chronic and older with an average age of 41 years at initial diagnosis.[54,56] ITP can be classified into two subgroups, primary and secondary. Primary ITP is the presence of thrombocytopenia without any other underlying cause, whereas secondary ITP is associated with viral infections, autoimmune disorders or malignant cancers.[57,58] ITP can also be classified as refractory depending on its severity of bleeding risk and resistance to treatment.[57,58] The cause of ITP is unknown, but it is often hypothesized a result from an immune cross-reactivity between bacterial or viral proteins bearing structural resemblance to platelet receptors.[59–61] As primary ITP has no other diagnostic criteria other then thrombocytopenia it can be difficult to confirm, and clinicians often rely on ruling out all other causes of thrombocytopenia before making a diagnosis.[62]

ITP treatment typically consists of immune suppression and while effective it is usually short term with significant side effects and costs associated with it. First-line treatment options for ITP patients are either corticosteroids or IVIG. Corticosteroids such as prednisone, dexamethasone and methylprednisone are common treatment options for ITP, however, relapses are frequent as sustained responses vary from 13% to 50% depending on the steroid used, its dosage and the follow up time period of the study.[58] Intravenous immunoglobulin (IVIG) and anti-D are also effective treatment options but the response is often temporary, lasting only a few weeks while the medication itself is expensive due to being derived from human plasma.[58,63]

Following the failure of first line treatment, secondary treatment options include TPO mimetics, splenectomy, and rituximab.[64] TPO mimetics such as eltrombopag and romiplostim are TPO agonists and stimulate platelet production. They are effective in raising platelet levels in ~70% of patients.[10,65] Despite this initial effectiveness, only 30% of patients retain a platelet response after TPO mimetic treatment is ceased.[66,67] This transient response along with the high cost of medication, estimated ~$1,200 per single use vial of romiplostim, limit the potential use of this medication.[63, 62,65] Splenectomy, removal of the spleen, has a high reported remission rate of 68% in ITP patients, however, the risks and costs associated with initial surgery as well as a lifetime risk of increased thrombosis and infection generally discourage patients from undertaking the procedure.[64,69] Rituximab and other less common drugs such as azathioprine and cyclosporin are also used, but excessive use compromises the immune system and exposes ITP patients to an increased risk of opportunistic infections.[58]

Given the risk of bleeding, frequent relapses and a compromised immune system, chronic ITP patients usually require constant monitoring by a clinician and experience an overall reduction in their quality of life.[70] Better understanding of ITP pathology could aid in both diagnosis, prognosis and the development of more effective treatments for ITP patients.

1.8 ITP autoantibodies and the pathology of platelet destruction

ITP is primarily caused by antiplatelet autoantibodies that bind and destroy circulating platelets. Historically the pathology of ITP was elusive until the experiments performed by Dr. Harrington demonstrated ITP plasma can induce severe thrombocytopenia when injected into healthy donors, including himself. [71] Fractionating ITP plasma determined this effect was originating from the antibody fraction. [72] These autoantibodies bind to platelet receptors and opsonize the platelet, signalling it for destruction by the immune system via complement activation and phagocytosis.[73] This destruction and clearance predominately occurs in the spleen by the RES.[73] Following this discovery, research shifted towards identifying and characterizing these ITP autoantibodies.

Numerous assays were developed to detect and characterize ITP autoantibodies, as this could be a useful diagnostic and prognostic tool. Early assays measuring total antibody binding to platelets were unsuccessful as the specificity was low and unable to separate ITP patients from other non-immune thrombocytopenic patients.[74] Through a combination of antigen capture assays and the use of platelets from genetic platelet disorders, research groups determined that ITP patients predominately possess anti-GPIIb/IIIa (CD41a) and anti-GPIb (CD42b) autoantibodies.[74,75] Autoantibodies of other platelet receptors such as GPIa/IIa, GPV, and GPIV were also detected but at lower

frequencies (<20%) and almost always in combination with either anti-GPIIb/IIIa or anti-GPIb autoantibodies.[75,76]

Several different antigen capture assays (ACA) have been developed to detect anti-GPIIb/IIIa or anti-GPIb autoantibodies in ITP patients, which can be classified as direct or indirect ACA.[74] Direct ACA use patient platelets that are lysed with the relevant receptors captured using monoclonal antibodies onto an enzyme linked immunosorbent assay (EIA) for detection of bound antiplatelet autoantibodies. Indirect ACA use patient sera or plasma and donor platelets to capture circulating antiplatelet autoantibodies and detect them on an EIA. In ITP, direct ACA have a higher sensitivity than indirect assays, meaning ITP autoantibodies were more often detected bound to platelets than in the circulating plasma.[74,77] There has been no reported correlation between the presence of an autoantibody and the degree of thrombocytopenia present in ITP. Assay development on improving screening for these antiplatelet autoantibodies remains ongoing.

Current ACA have low sensitivity and high specificity for the detection of antiplatelet autoantibodies in ITP patients. Direct ACA assays can detect anti-GPIIb/IIIa autoantibodies in 48% of patients and 37% for anti-GPIb, with specificity being 92% and 98% respectively. Indirect assays have a sensitivity of 24% and 8% for anti-GPIIb/IIIa and anti-GPIb antibodies.[77] Analysis of the antigen binding locations suggests some of these autoantibodies could be irrelevant as they bind to cytoplasmic regions of the platelet receptors and likely arise as a by-product of previous platelet destruction. This destruction

would expose new antigenic targets for autobody development.[78] The low frequency of antiplatelet autoantibodies detected in ITP patients indicates other pathological mechanisms must be involved and that ITP is likely a heterogeneous disorder with multiple pathologies.[73] In addition to antiplatelet autoantibodies, autoreactive cytotoxic T cells, loss of T regulatory cell and various inflammatory cytokines imbalances have been detected in ITP patients, but their relevance in pathology remains undetermined.[79–81]

1.9 Platelet receptor GPIIb/IIIa

Anti-GPIIb/IIIa autoantibodies are the most prevalent autoantibodies detected in ITP patients, where its target antigen GPIIb/IIIa functions as a key receptor for platelet aggregation.[77,82] GPIIb/IIIa (αIIbβ3, CD41a, CD41/61) is the most prevalent receptor expressed on platelets with an estimated 80,000 copies present on the surface.[83] This transmembrane receptor is a heterodimer composed of two integrin proteins: a two peptide chain of GPIIb (116 kDa and 23 kDa) and a single chain GPIIIa (108 kDa).[84] The two integrins bind together and remain in an inactive conformation until platelet activation, where internal signalling results in the cytoplasmic tails uncoupling causing a conformation change in the receptor and increasing its affinity for various plasma proteins, most notably fibrinogen.[85] Fibrinogen is a soluble protein present at high concentrations in blood and functions as a key substrate for coagulation.[86] Activated GPIIb/IIIa recognizes at least three different binding sites on fibrinogen (RGD and HHLGGAKQAGDV peptide sequences) thereby allowing multiple receptors to bind to

the same molecule.[87] This multiple binding to fibrinogen crosslinks GPIIb/IIIa receptors on different platelets, causing them to aggregate and help form a platelet plug.[87] Clustering of activated GPIIb/IIIa receptors on a platelet surface also activates several signalling kinases, which generate further signalling that supports internal platelet activation.[88] In addition to fibrinogen, GP IIb/IIIa can also bind VWF, fibronectin, and vitronectin amongst others.[87] On megakaryocytes, CD41a is the earliest indicator of megakaryopoiesis and exposure to fibrinogen is known to promote thrombopoiesis on mature megakaryocytes.[35,89] Loss of expression of this receptor, such as in Glanzmann's thrombasthenia is associated with defective platelet aggregation and a prolonged bleeding time.[90]

1.10 Platelet receptor GPIb

GPIb is part of the GPIb/IX/V signalling complex involved in platelet adherence and activation at the site of vascular damage and is the second most frequent antiplatelet autoantibody target detected in ITP patients.[77,91] Also known as CD42, GPIb has approximately 20,000-50,000 copies expressed on the platelet surface. It is a heterotrimer comprised of two GPIbβ (25 kDa) subunits bound to a single GPIbα (135 kDa) subunit connected by two disulphide bonds. [92–94] The GPIb/IX/V complex recognizes multiple different ligands including von VWF, α-thrombin, p-selectin, thrombospondin, Mac-1, Factor XI, Factor XII, and Kininogen.[91,94,95] Exposure to unravelled VWF, which is considered the main ligand, helps platelets adhere at the site of vascular damage and initiate signalling events facilitating platelet activation and thrombosis.[91] This receptor

complex is attached to the actin cytoskeleton via filamin and activates multiple different signalling pathways including 14-3-3 ζ, calmodulin and PI3K with others currently under investigation.[95] GPIb is also expressed on megakaryocytes and is considered an indicator of megakaryocyte maturation.[40]

1.11 Impaired platelet production in ITP patients

As megakaryocytes express platelet receptors, the potential for ITP autoantibodies to inhibit platelet production was investigated as a mechanism of causing thrombocytopenia. Platelet survival studies in ITP patients demonstrated one third of ITP patients had reduced capacity to produce platelets, one-third had production levels comparable to healthy donors and the remaining third had increased production to compensate for platelet destruction.[96,97] Early microscopy studies suggested some cellular damage was occurring on individual megakaryocytes in the bone marrow.[98] The inhibition of platelet production by antiplatelet autoantibodies would explain why ITP patients have thrombocytopenia while testing negative or weakly positive for autoantibodies as the effect would be localized within the bone marrow

Several studies tested ITP sera and purified antibodies for inhibition of megakaryopoiesis using in-vitro megakaryopoiesis cell cultures. The result of those studies demonstrated some ITP plasma inhibited megakaryocyte growth and the effect was partly antibody mediated.[99–102] Some of this inhibition was plasma factor dependent,

suggesting complement activity, and some was plasma factor independent with inhibition present when testing the purified IgG antibody fraction.[99,100,103] Additional studies investigating thrombopoiesis demonstrate some ITP plasma samples cause deformities in proplatelet extensions on mature megakaryocytes which could be inhibiting thrombopoiesis.[104,105] Despite these findings, no correlation has been reported between the inhibition of megakaryopoiesis or thrombopoiesis and the presence of an antiplatelet autoantibody, and no mechanism has been determined on how antiplatelet autoantibodies cause inhibition. These results are further complicated as they were conducted using non-autologous culture systems, mixing patient samples with healthy donor HSPC undergoing megakaryopoiesis.[99,100] Platelets and megakaryocytes express blood type antigens, human leukocyte antigens (HLA), as well as platelet leukocyte antigens that are known to induce an antibody immune response. [106–108] HLA antibodies have demonstrated the ability to activate platelets, cause aggregation and cause non-specific effects.[109,110] These factors could obfuscate results making it difficult to identify and characterize antibody mediated inhibition of megakaryopoiesis. Recently, anti-TPO and anti-CMpL autoantibodies have also been detected in ITP plasma with some evidence to suggest they can cause inhibition of megakaryopoiesis.[111,112] However, these antibodies have also been found in other autoimmune disorders in which impaired platelet production is not suspected to be a pathology. The current implications of this finding is unclear.[112]

Several ITP observations are also inconsistent with this impaired platelet production theory indicating further investigation is required. Direct bone marrow

biopsies of ITP patients show most patients have megakaryocyte levels comparable to healthy donors and only 56% of patients have detectable antiplatelet autoantibodies in the bone marrow niche.[113,114] Mouse studies in which anti-platelet antibodies are injected into murine models also cause transient thrombocytopenia, but no change in megakaryocyte numbers in the bone marrow.[115] TPO mimetics which mimic TPO function and stimulate platelet production are successful in treating some ITP patients.[10] The success of mimetics as a treatment raises questions about why the body is not naturally increasing platelet production to support homeostasis. TPO concentrations in ITP plasma samples are not elevated as in the case with other thrombocytopenic patient populations.[116] All these results suggest that impaired platelet production could result from a lack of stimulation and not a direct consequence of inhibited megakaryopoiesis. The role of antibody mediated inhibition of megakaryopoiesis in ITP pathology remains to be determined.

1.11 Thesis Objectives

The last few decades of ITP research have demonstrated that there is impaired platelet production occurring in ITP. But whether this pathology is caused by antibody mediated inhibition of megakaryopoiesis remains unclear. Previous studies demonstrate there could be inhibition of megakaryopoiesis, but its prevalence and mechanisms remain unknown and non-autologous factors make conclusions difficult to confirm. Positive controls such as a reliable inhibiting monoclonal antibody that can replicate inhibition

have not been identified and as such any mechanism of inhibition cannot be dissected and identified. As a result, more research is needed to establish if there is any inhibition of platelet production and determine its relevance in ITP pathophysiology, which was the aim of this thesis project.

We hypothesize that a subset of ITP patients possesses antiplatelet autoantibodies that are capable of inhibiting megakaryopoiesis. Confirming this hypothesis would clarify previous studies and be used to identify both the mechanisms of inhibition, its prevalence in the ITP population and determine if any clinical usefulness exists in identifying these inhibiting autoantibodies. To address this hypothesis, I have three objectives:

1) Develop an autologous megakaryopoiesis assay that can be used to screen for antibody mediated inhibition. This assay must be capable of using ITP patient HSPC to grow mature megakaryocytes for study.

2) Use monoclonal antiplatelet antibodies against cells undergoing megakaryopoiesis to demonstrate inhibition of megakaryopoiesis. This goal would identify any reliable positive controls, confirm inhibition is present, and be used as a tool to study its mechanism.

3) Test ITP patient plasma in an autologous megakaryopoiesis assay to identify any inhibition of megakaryopoiesis. These results would confirm if there is any inhibition and will be analyzed with patient clinical information to determine any clinical significance.

The results of these objectives will be summarized as separate chapters in this thesis. There will be some overlap between chapters as similar methods are used in different studies and some chapters are reprints of manuscripts.

Chapter 2:

Developing a peripheral blood based megakaryopoiesis assay

Reprinted from Transfusion, Vol. 56 (5), Nikola Ivetic[1], Ishac Nazy[2], Nadia Karim[2], Rumi Clare[2], James Smith[2], Jane Moore[2,3] Kristin Hope[1], John G. Kelton[2], Donald M. Arnold[2,4]. Producing Megakaryocytes from a Human Peripheral Blood Source, 1066-74,

2.1 Author's preface

This chapter is focused on the development of a peripheral blood based megakaryopoiesis assay. The establishment of an in-vitro megakaryopoiesis assay is necessary as this allows investigations into ITP impaired platelet production to commence. This cell culture was designed to use relatively small amounts of peripheral blood directly from ITP patients as a source of hematopoietic stem and progenitor cells; thereby making these studies completely autologous. Another benefit of this cell assay is that it can be used on any patient at any time point as peripheral blood is easily accessible and readily available. A major limitation of using peripheral blood is the small yield of hematopoietic stem and progenitor cells that can be isolated for culture. To overcome this limitation an expansion procedure was used to increase the initial HSPC population. We then demonstrate that these expanded cells can be stimulated with TPO and undergo megakaryopoiesis, developing into mature megakaryocytes with all known megakaryocyte features including the capability of undergoing thrombopoiesis. NI designed and performed all cell culture experiments and all flow analysis with assistance from Nadia Karim and Rumi Clare. James Smith and Jane Moore provided technical advice. Marcia Reid from the Faculty of Health Science's Electron Microscopy Facility helped develop the processed samples for imaging. NI analyzed the data and prepared the figures and wrote the manuscript. Dr. I. Nazy, Dr. K. Hope, D.M. Arnold, Dr. J.G. Kelton helped with editing.

2.2 Abstract

Background: Cultured megakaryocytes could prove useful in the study of human diseases, but it is difficult to produce sufficient numbers for study. We describe and evaluate the use of an expansion process to develop mature megakaryocytes from peripheral blood-derived human hematopoietic stem and progenitor cells (HSPCs).

Study Design and Methods: HSPCS ($CD34^+$) were isolated from peripheral blood by positive selection and expanded using an optimal $CD34^+$ expansion supplement. We evaluated megakaryocyte growth, maturation, and morphology in response to thrombopoietin (TPO) stimulation using flow cytometry and electron microscopy. TPO demonstrated a dose-dependent stimulatory effect on both megakaryocyte number and maturation.

Results: From 90–120 ml of unmanipulated peripheral blood, we isolated an average of 1.5×10^5 HSPCS (1.5×10^3 cells per ml of whole blood). HSPCSs expanded 9-fold after a 4-day culture using an expansion supplement. Expanded cells were cultured for an additional 8 days with TPO (20 ng/ml) which resulted in a 2.9 fold increase in megakaryocytic cells where 83% of live cells expressed $CD41a^+$, a marker of megakaryocyte commitment, and 50% expressed $CD42b^+$, a marker for megakaryocyte maturation. The expanded HSPCSs responded to TPO stimulation to yield $> 1.0 \times 10^6$ megakaryocytes. This cell number was sufficient for morphological studies that demonstrated these expanded HSPCSs produced mature polyploid megakaryocytes capable of forming proplatelet extensions.

Conclusions: Peripheral blood HSPCSs can be expanded and differentiated into functional, mature megakaryocytes; a finding that supports the use of this process to study inherent platelet production disorders as well study factors that impair normal platelet production.

2.3 Introduction

Platelets are produced from megakaryocytes in the bone marrow. Megakaryocytes, in turn, are continually regenerated from hematopoietic stem and progenitor cells (HSPCs). This process, called megakaryopoiesis, is regulated by a number of cytokines with the most important being thrombopoietin (TPO).[1,2] Stimulation with TPO results in HSPC commitment to the megakaryocyte lineage. During megakaryocyte maturation, certain platelet glycoproteins are expressed with glycoprotein (GP) IIb/IIIa (CD41a) initially, followed by GP Ib (CD42b).[3] As megakaryocyte progenitor cells lose the ability to proliferate they undergo endomitosis to produce a polyploid nuclei, develop platelet organelles and form a demarcated membrane system (DMS).[1] As they complete maturation, megakaryocytes migrate to bone marrow sinusoids where they extend pseudopod-like proplatelet extensions into circulation and release newly-formed platelets.[1,4]

Several inherited and acquired thrombocytopenic syndromes are characterized by impaired platelet production.[5–7] To better understand the mechanisms of these disorders, a megakaryopoiesis model is needed. Studying human megakaryocytes is difficult since these cells are sequestered in the bone marrow and are therefore difficult to access. Furthermore these cells are rare, comprising 0.03% of the bone marrow cell population. [8] As a result, research on megakaryopoiesis relies mostly on animal models or on human HPSC sources from bone marrow, cord blood or peripheral blood stimulated with granulocyte colony stimulating factor (“mobilized blood”) and subsequently cultured into megakaryocytes. [9–11] These HSPCs sources are difficult to access and have limited use in

direct patient studies, which restricts their applications. Therefore, an alternate method of studying human megakaryopoiesis that will be sensitive to both normal and disease-compromised development of functional and morphologically mature megakaryocytes is needed.

The use of peripheral blood as a source of HSPCs may overcome limitations of other megakaryopoiesis models. Peripheral blood is easily accessible, requiring minimal invasive procurement for the collection of HSPCs, and allows direct analysis of patient developed megakaryocytes. Peripheral blood has been used to study megakaryopoiesis and inherent platelet production disorders; however, the main challenge with using peripheral blood derived $CD34^+$ HSPCs is that these cells are rare, comprising less than 0.1% of all nucleated cells in circulation.[6–8,12–17] Directly isolating peripheral blood derived HSPCs and culturing them into megakaryocytes is slow, labor-intensive and simply does not produce enough megakaryocytes for practical investigations. As a result, the majority of current peripheral blood megakaryopoiesis assays either rely on apheresis procedures or unpurified peripheral blood monocular cells (PBMCs) for megakaryopoiesis.[7,12,18] To improve yields, these assays often combine TPO with other supportive cytokines to increase the number of megakaryocyte progenitors generated as the cells undergo megakaryopoiesis.[8,19]

Rather than follow this approach, we investigated if increasing HSPCs numbers before stimulating megakaryopoiesis would be a better alternative. By expanding HSPCs rather than megakaryocyte progenitors, we retain when and under what conditions cells begin megakaryopoiesis-an area that may be of interest to investigators- while still

overcoming the low yield problems associated with peripheral blood. This approach retains all the benefits of using peripheral blood while being a clean system that does not require additional equipment or procedures common in other techniques such as apheresis. Such an approach has not been applied to peripheral blood derived HSPCs for megakaryopoiesis studies and so in this report, we describe and evaluate the HSPC expansion technique to increase the number of HSPCs obtained from peripheral blood. We then evaluated, using electron microscopy and immunophenotyping via flow cytometry, the derived megakaryocytes from those expanded cells.

2.3 Material and Methods

Isolation of HSPCs from Peripheral Blood

Peripheral blood (90-120 ml) was collected from healthy donors into sterile acid citrate dextrose (ACD) anticoagulant containing 1 mM theophylline and 3 μM aprotinin at a 6:1 blood to anticoagulant ratio. The blood was depleted of platelet rich plasma following differential centrifugation (150g, 20 minutes) and removal of the top platelet rich plasma fraction. The remaining blood was then diluted two-fold with isolation wash buffer, that is composed of sterile phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic (EDTA) acid, 1 mM theophylline, 0.15 μM aprotinin, and 0.02 mM prostaglandin E1. The diluted blood was then layered on Histopaque (cat 1077, Sigma-Aldrich, St. Louis USA) at a 2:1 ratio (30 ml of blood to 15 ml of Histopaque) and centrifuged for 30 minutes at 400g. The peripheral blood mononuclear cells (PBMCs) were then collected, diluted five fold and

centrifuged at 370 g for 10 minutes as a wash. The washed PBMC cell pellet was suspended to a final volume of 25 ml in isolation buffer before layering on 15 ml of Optiprep density gradient to deplete contaminating platelet levels as previously published; Optiprep density was 1.063 g/ml and the sample was spun at 350 g for 15 minutes. (Optiprep, Axis-Shield, Oslo Norway)[13,20] The resulting cell pellet was then diluted to 50 ml with isolation wash buffer and centrifuged at 200g for 12 minutes as a wash. HSPCs ($CD34^+$) were then isolated from PBMC using the StemSep human $CD34^+$ positive selection kit (cat 18056, StemCell Technologies, Vancouver, Canada) via a magnetic separation column as per manufacturer's instructions (cat 130-090-976 QuadroMac Separator, cat 130-042-401 Multistand, cat 130-042-401 Separation Columns, cat 130-041-407 Pre-separation filters all from Miltenyi Biotec, Bergisch Gladbach, Germany) This study was done with approval from the Hamilton Integrated Research Ethics Board and all samples were obtained with informed, written consent.

Once isolated, cells were analyzed for CD34 and CD41a expression using flow cytometry, while viability and the absolute cell count was determined using a TC-20 automatic hemocytometer (cat 145-0102 Bio-Rad, Hercules, USA) or the Countess Automated Cell Counter (c10227 Thermo-Fisher Scientific, Waltham USA).

Expansion of HSPCs

Isolated HSPCs were suspended in culture media containing Iscove's Modified Dulbecco's Medium containing GlutaMax (cat 31980030 Life Technologies Carlsbad USA), supplemented with BIT-9500 serum substitute (15% v/v, cat 09500 StemCell

Technologies), 100 units/ml of penicillin, and 100 µg/ml of streptomycin. The cells were suspended to a final concentration of $5.0x10^4$ HSPCs ($CD34^+$) per ml prior to expansion.

Isolated HSPCs were expanded using the StemSpan $CD34^+$ Expansion Supplement that contains recombinant human TPO, interleukin-3, interleukin-6, FMS-related tyrosine kinase 3 and stem cell factor along with additional proprietary additives (cat 02691StemCell Technologies). The HSPCs were seeded in 1 ml aliquots in culture media with the expansion supplement at a 10% v/v ratio using 24-well culture plates. The cells were expanded for four days at 37˚C and 5% CO_2 or until culture confluency reached over 80% as assessed by light microscopy. This four-day time period was chosen since it resulted in an expansion of HPSCs that was in the range reported for the supplement. Preliminary data indicated a loss of viability and CD34 expression at longer expansion times (data not shown). Cell counts, CD34 expression, and viability were measured, and the fold increase of HSPCs was calculated as the absolute number of $CD34^+$ cells obtained after expansion divided by the initial HSPC seeding population. Absolute HSPC counts were calculated as the product of the percentage of live cells expressing CD34 and the total live cell count, which were determined through the use of flow cytometry and an automated hemocytometer (TC-20 Bio-Rad or Countess Invitrogen). CD41a and CD42b expression was also evaluated after expansion.

Megakaryocyte growth and maturation of expanded cells

Expanded $CD34^+$ cells were washed and suspended in fresh culture media (5.0 x 10^4 $CD34^+$ cells /ml) before being seeded in triplicate 200 µL wells in a 96-well plate.

The fresh culture media was identical to the expansion media with the exception of replacing the expansion supplement with varying concentrations of recombinant TPO (0, 0.25, 0.5, 1, 10, 20, 40 ng/ml) (cat 288-TP, R&D Systems, Minneapolis, USA) and cultured for 8 days. Since TPO is added to expanded HSPCs, and this begins megakaryopoiesis, we defined this time point as day 0 or baseline for subsequent megakaryopoiesis analysis; the isolation and expansion of HSPCs is treated as a separate process. Megakaryocyte growth and maturation status were evaluated by measuring the expression of CD41a and CD42b (See flow cytometry analysis). The absolute number of megakaryocytes was determined as the product of the live cell count and the percentage of cells that were $CD41a^+$. The absolute number of mature megakaryocytes was calculated as the product of the live cell count and the percentage of cells that were $CD41a^+CD42b^+$. Cell counts were normalized to the baseline total cell seeding population (day 0) to determine the fold increase of specific cell populations.

To determine the optimal megakaryocyte yield in this assay, expanded cells were suspended in fresh media supplemented with 20 ng/ml of TPO, which was determined to be a saturating amount based on the TPO dose results, and analyzed for $CD41a^+$ and $CD41a^+$ $CD42b^+$ expression on days 0 (Baseline), 6, 8 and 10 of culture. The total cell count was recorded, and the absolute number of megakaryocytes was determined as the product of the live count and the percentage of cells that were $CD41a^+$ as stated previously and normalized to the initial cell seeding population. Identical calculations were performed to determine the increase in the mature megakaryocyte population

($CD41a^+$ $CD42b^+$). Day 8 cultured megakaryocytes were assessed for the presence of DNA polyploidy (see flow cytometry analysis).

Flow Cytometry Analysis

All antibodies and flow cytometry stains were obtained from BD Biosciences (Franklin Lakes, USA) with the exception of monoclonal TW-1 (antibody against CD42b) which was developed in house. Isolated HSPCs were labelled with fluorescein isothiocyanate (FITC) conjugated CD45 monoclonal antibody and phycoerythrin-cyano 5 (PECy5) conjugated CD34 monoclonal antibody. HSPCs were identified as $CD45^+/CD34^+$ and purity calculated as the percentage of $CD34^+$ events within the $CD45^+$ gate.[21] Following expansion, cells were assessed for CD34 expression using the same CD34-PECy5 monoclonal antibody. Appropriately matched isotype controls were used to establish background fluorescence for subsequent positive identification.

CD41a-FITC and CD42b-Phycoerythrin (PE) monoclonal antibodies and properly matched isotypes were used to identify megakaryocytes ($CD41a^+$) and their maturation status ($CD41a^+$ $CD42b^+$). Annexin-V and propidium iodide staining was used to determine the scatter characteristics of dead cells and used to excluded them from analysis.[22] Megakaryocyte ploidy was assessed by a double staining technique as previously published.[23] Briefly, cultured cells were suspended in PBS supplemented with 1% bovine serum albumin and stained with a monoclonal FITC conjugated antibody against CD41a. Labelled cells were fixed with 0.5% paraformaldehyde for 45 minutes at room

temperature, washed with PBS and incubated overnight with DNA staining solution (PBS with 2 mM $MgCl_2$, 0.05% Saponin, 10 µg /ml propidium iodide and 10 units/ml of RNAse A) (St Louis USA, Sigma Aldrich). $CD41a^+$ cells were identified using isotype controls and the initial 2N peak using a control sample of lymphocytes; subsequent peaks were identified as polyploid cells. To clearly demonstrate the presence of DNA ploidy amongst mature megakaryocytes, a representative culture was also stained with TW-1 FITC (antibody against CD42b) and propidium iodide. All flow cytometry data was collected using a Beckman Coulter Epic XL-MCL and subsequent analysis performed using FlowJo software (Version 7.6, Ashland, USA).

Transmission electron microscopy

To assess the development of megakaryocyte morphology, expanded HSPCs were seeded at 5.0 x 10^4 $CD34^+$ cells /ml in a 5 ml well of a 6 well culture plate with 20 ng/ml of TPO and cultured for days 0, 6, 8 and 10 before being fixed and analyzed via transmission electron microscopy. Fixation was performed with 2% Glutaraldehyde (v/v) in 0.1 M PBS (pH 7.4). The cells were then rinsed twice in PBS (800 g, 2 minutes), and post-fixed with 1% osmium tetroxide (in PBS) for 1 hour. The samples were then dehydrated through a graded ethanol (EtOH) series (50%, 70%, 70%, 95%, 95%, 100%, 100%). Following dehydration, infiltration with Epon resin was performed through a graded series (2:1 EtOH:Epon, 1:1 EtOH:Epon, 1:2 EtOH:Epon, 100% Epon (3x)) with rotation of the samples in between solution changes. The samples were then transferred to embedding molds that were then filled with fresh Epon resin and polymerized overnight

in a 60°C Isotemp oven (Thermo-Fisher Scientific). Thin sections were cut on a Leica UCT Ultramicrotome (Leica Microsystems, Concord, Canada) and picked up onto copper grids. The sections were post stained with uranyl acetate (50% EtOH)and Reynold's lead citrate and then viewed in a JEOL JEM 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, USA) operating at an accelerating voltage of 80kV.

Scanning electron microscopy

Expanded HSPCs were seeded at $5.0x10^4$ $CD34^+$ cells /ml in 1 ml wells of a 24 well plate and cultured with a saturating TPO concentration (20 ng/ml). On days 0, 6, 8 and 10, cultured cells were transferred onto fibrinogen coated chambered slides (Nunc™ Lab-Tek™ II Chamber Slide™, cat 154526, Thermo Scientific) and incubated overnight at standard culture conditions. Coating of chambered slides with fibrinogen was performed as follows; chambers were incubated overnight with a fibrinogen solution (100 µg/ml of fibrinogen in PBS, cat FIB2 Enzyme Research Laboratories, South Bend USA) at 37°C, the chamber was then washed twice using PBS and blocked with a 2% BSA/PBS solution for 1 hour at 37°C, washed again twice with PBS and air dried (room temperature). All steps were performed under sterile conditions.

Following overnight cell incubation, cells were fixed with 2% Glutaraldehyde in 0.1 M PBS (pH 7.4). Sample processing was similar to the TEM protocol, except after dehydration in 100% EtOH, samples were critical point dried, mounted onto placement stubs, sputter-coated with gold and then viewed in a Tescan Vega II LSU scanning

electron microscope (Tescan, Libušina, Czech Republic) operating at 20kV. Cells were directly observed for the presence of proplatelet formation.[4]

2.4 Results

Isolation and Expansion of HSPCs from Peripheral Blood

From peripheral blood samples (n=11) the purity of HSPCs ($CD34^+$) isolated was 82 ± 17%, viability was 79 ± 11% and the yield was 1.5 x 10^5 cells (1,500±800 HSPCs per ml of blood) (mean ±SD). During expansion, the HSPCs ($CD34^+$) population increased by 9.2±3.2 fold while maintaining cell viability and $CD34^+$ expression (Table 1). This CD34 expression level both before and after expansion is comparable to that reported in previous publications.[12,13,18] Initial CD41a expression of isolated HSPCs was 2±1% (n=4), while post-expansion expression was 23±12% (n=7), indicating some level of megakaryopoiesis occurred during expansion (mean ±SD).

Megakaryocyte Growth and Maturation

Megakaryopoiesis from expanded peripheral blood derived HSPCs was TPO dose dependent as shown by progressive increases in CD41a and CD42b expression with optimal growth at concentrations ≥ 10 ng/ml of TPO (Figure 2.1). The proportion of cells that were $CD41a^+$ and $CD41a^+$ $CD42b^+$ increased from 17±8% and 1±1% without TPO, to 83±2% and 52±11% respectively at 40 ng/ml of TPO (Figure 2.1) (mean ± SD). Although 40 ng/ml of TPO resulted in the highest amount of CD41a and CD42b expression its results are identical to the 10 and 20 ng/ml findings; where $CD41a^+$

expression was 83±4% and 83±5% while $CD41a^+$ $CD42b^+$ expression was 48±11% and 50±8% of the total cell population (mean ± SD), respectively. These results indicate that 10 ng/ml of TPO is a saturating amount and that higher TPO concentrations have a minimal impact on megakaryopoiesis. Based on flow cytometry forward and side scatter properties, cells cultured without TPO or with TPO at concentrations that were lower than 10 ng/ml were smaller and less granular than cells cultured with higher TPO concentrations (data not shown). The low level of $CD41a^+$ and $CD42b^+$ expression from the 0 ng/ml TPO concentration indicates that the majority of megakaryopoiesis is a result of the TPO added after HSPCs expansion; indicating that the level of megakaryopoiesis from those expanded HSPCs can be directly controlled by the amount of TPO added. As a result of these findings, 20 ng/ml of TPO was chosen as a saturating concentration for subsequent experiments

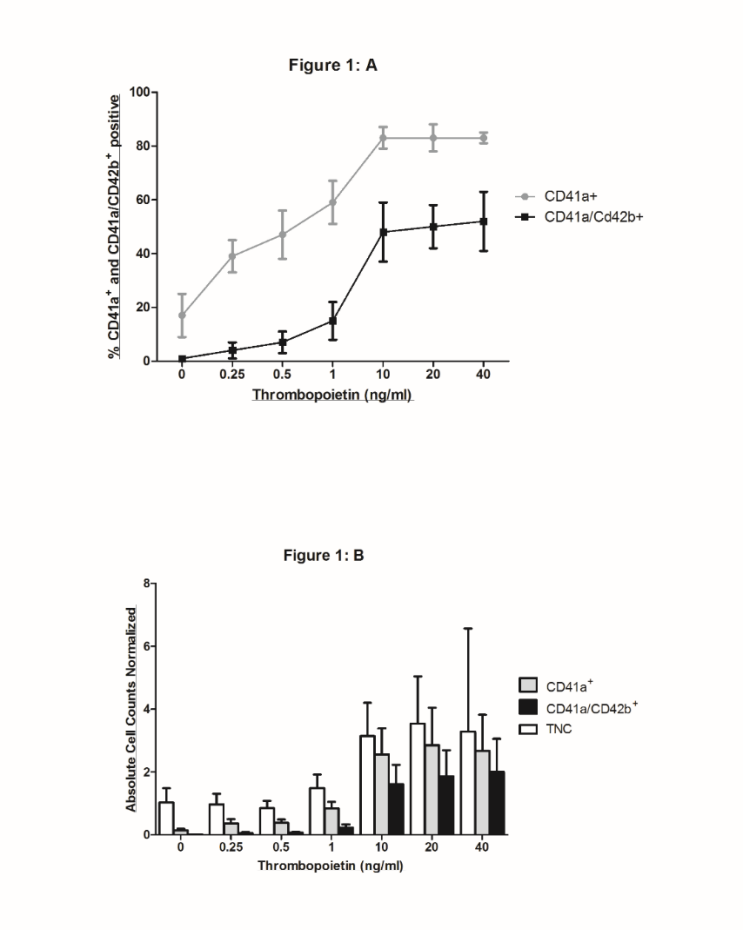

Figure 2.1: Expanded HSPCs undergo megakaryopoiesis following TPO stimulation with maximal commitment occurring at concentrations ≥10 ng/ml. A) Expanded HSPCs were stimulated with varying concentrations of TPO (0-40 ng/ml) and evaluated for the expression of CD41a and then CD42b. A TPO stimulatory response inducing megakaryopoiesis is evident in which the level of cells expressing $CD41a^+$ increased from 17±8% to 83±4% at 10 ng/ml before it plateaued. Concurrent $CD41a^+$ $CD42b^+$ expression also increased from 8±3% to 48±11% of the total live cell population at 10 ng/ml. Of the $CD41a^+$ fraction, 62±17% of cells were $CD42b^+$. TPO concentrations above 10 ng/ml had little to no impact on level of megakaryopoiesis. Data represented as the mean ± SD of three separate experiments **B)** The same trends were detected when comparing absolute live cell counts; total nucleated cell growth (TNC) (3.1±1.1 fold), $CD41a^+$ (2.6±0.8 fold) and $CD41a^+$ $CD42b^+$ (1.6±0.6 fold) reached maximal growth before plateauing after 10 ng/ml. Data represented as the mean ± SEM of three separate experiments with the results normalized to the initial seeding population.

Following saturating TPO stimulation, megakaryocyte growth, as measured by the normalized absolute cell count, progressively rose from days 0 to 6, peaked on day 8 and began to decline by day 10 (Figure 2.2). Both total cell growth and megakaryocyte yield peaked on day 8 with a mean total nucleated cell increase of 3.5-fold and a mean megakaryocyte ($CD41a^+$) increase of 2.9-fold. Absolute $CD41a^+$ $CD42b^+$ fold increase was 1.5. In addition, the highest percentage of cells undergoing megakaryopoiesis was observed on day 8, where 83% of all live cells were $CD41a^+$ and 50% were $CD41a^+$ $CD42b^+$. When analyzing the $CD41a^+$ population for DNA polyploidy on day 8: 69±6% were 2N, 20±2% were 4N, 6±3% were 8N, and 4±4% were 16N or higher (mean ± SD of 3 separate experiments). Clear evidence that the cultured cells contain 34 N was detected when analyzing the mature megakaryocyte population (Figure 2.3). Cell viability decreased following expansion, dropping from 89±3% at baseline to 63±11% on day 6, to 57±14% on day 8, and 43±10% on day 10. The initial drop from day 0 to day 6 is likely the result of the removal of expansion cytokines that support cell viability, and this is then later on followed by the death of mature megakaryocytes. By day 8, the culture reached the limits of its growth and by day 10 the absolute number of megakaryocytes had begun to drop from 2.9-fold to 2.2-fold, while $CD41a^+$ and $CD41a^+$ $CD42b^+$ culture composition changed from 83% and 52% to 70% and 34% respectively (Figure 2.2). Thus, 8 days of TPO stimulation was determined to be the optimal growth period for these peripheral blood derived expanded HSPCs.

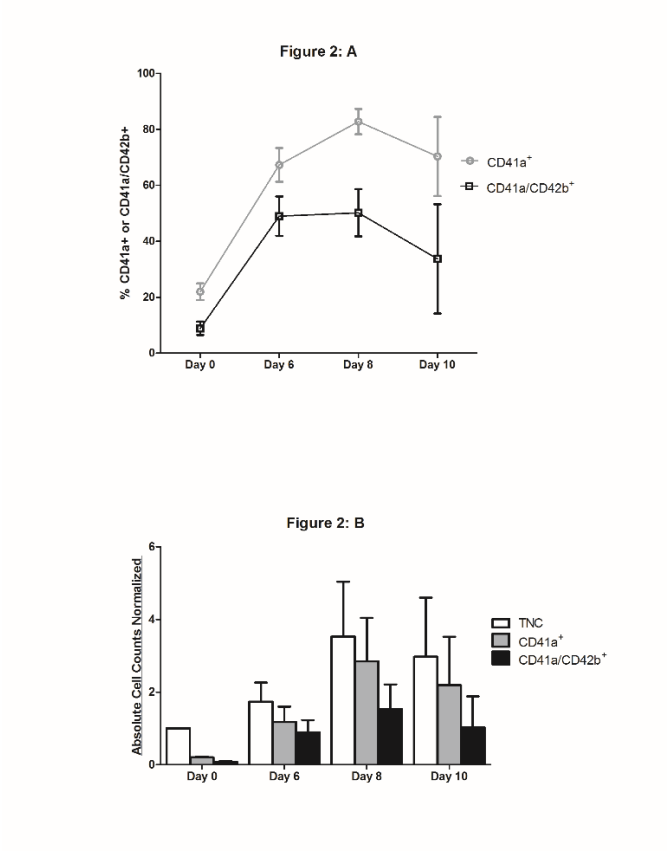

Figure 2.2: Maximal megakaryocytic growth is achieved after 8 days of TPO simulation. A) Expanded cells were stimulated with 20 ng/ml of TPO and analyzed for $CD41a^+$ and $CD41a^+$ $CD42b^+$ expression. (mean±SD, n=3) **B)** Absolute counts of total live cells, $CD41a^+$ and $CD41a^+$ $CD42b^+$ cells were also calculated and normalized to the initial cell seeding concentration. Maximal megakaryocytic growth was detected on day 8 with 83±5% of cells expressing CD41a and 50±8% of the total cell population was $CD41a^+$ $CD42b^+$; 61±13% of CD41a cells were $CD42b^+$. Mean fold increase was 2.9±1.2 and 1.5±0.7 for $CD41a^+$ and $CD41a^+$ $CD42b^+$ cells respectively (mean ± SEM, n=3).

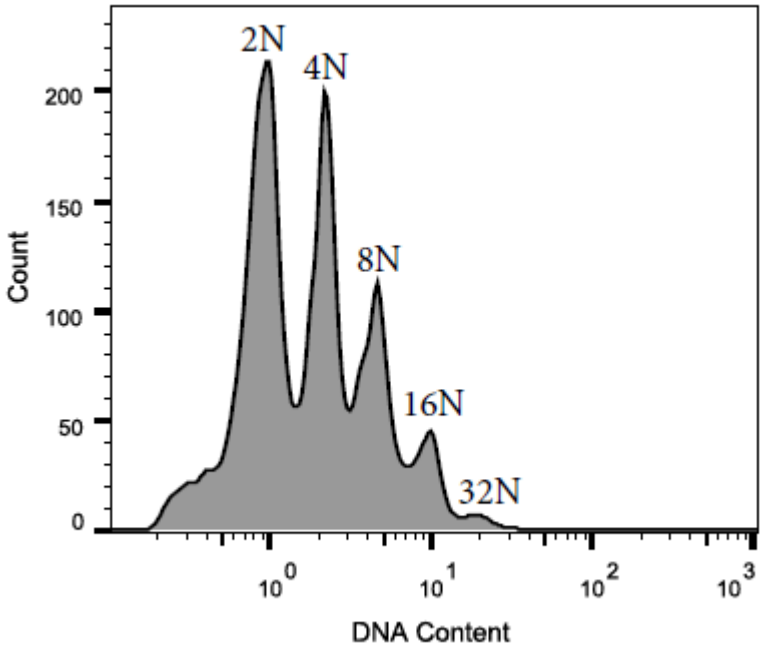

Figure 2.3: Cultured mature megakaryocytes demonstrate nuclear polyploidy
Expanded HSPCs were cultured for 8 days with 20 ng/ml of TPO and the DNA content of the mature megakaryocyte population **(**$CD42b^+$) was detected using propidium iodide; as shown, DNA content representing 2N to 32 N is clearly evident amongst the mature megakaryocyte population indicating polyploidy. A control lymphocyte population was used as a reference to identify the 2N population.

Megakaryocyte morphology and proplatelet formation

TEM images demonstrated that the expanded cells were developed into morphologically identifiable megakaryocytes (Figure 2.4).[1,24] On day 0, cultured cells were small, mononuclear and possessed few cellular organelles. By day 6, the cells had increased in size and granularity and evidence of a developing demarcation membrane system was present. By days 8 and 10, nuclear polyploidy was evident, a demarcated membrane system was present, and cells possessed extensive granulation and increased cytoplasmic size, indicative of platelet organelle formation.

SEM pictures demonstrated that cultured megakaryocytes from days 8 and 10 were able to form proplatelet extensions; indicating they were capable of generating platelets (Figure 2.5). These proplatelet extensions were also detected on day 8 during regular cell culture through the use of bright-field microscopy (data not shown).

These results demonstrate that expanded peripheral blood derived HSPCs had developed into identifiable and morphologically mature megakaryocytes that possessed the ability to form proplatelet extensions.

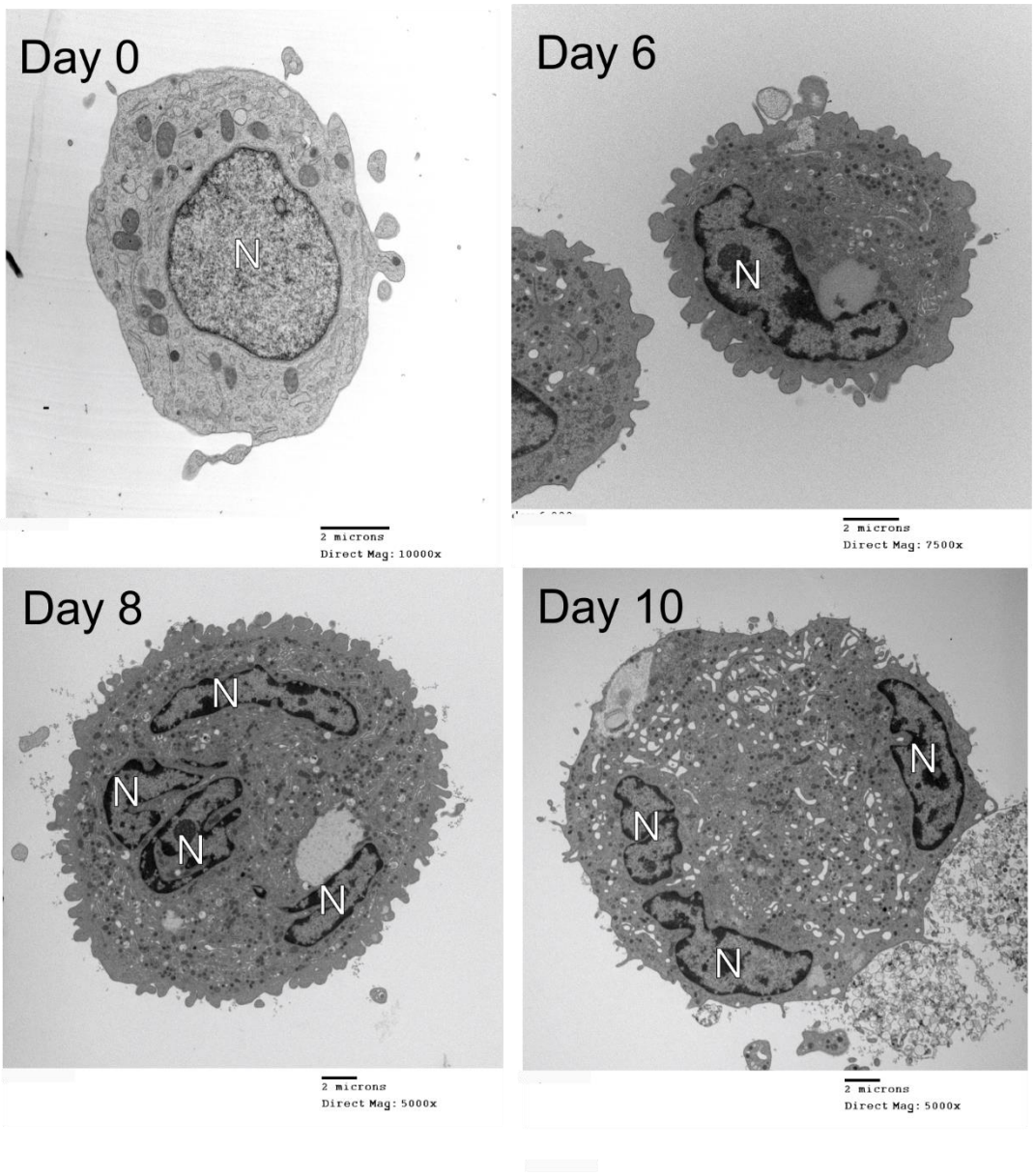

Figure 2.4: Transmission electron microscopy images reveal the development of megakaryocyte morphology
Expanded HSPCs were fixed, stained and analysed for megakaryocyte morphology on days 0, 6, 8 and 10 following TPO stimulation (20 ng/ml). The development of a polyploid nucleus is present by day 8 as shown by the multiple nuclei present (N). The demarcation membrane system is evident by day 6 and increases, along with cell size and granularity, as the megakaryocyte progenitors develop.

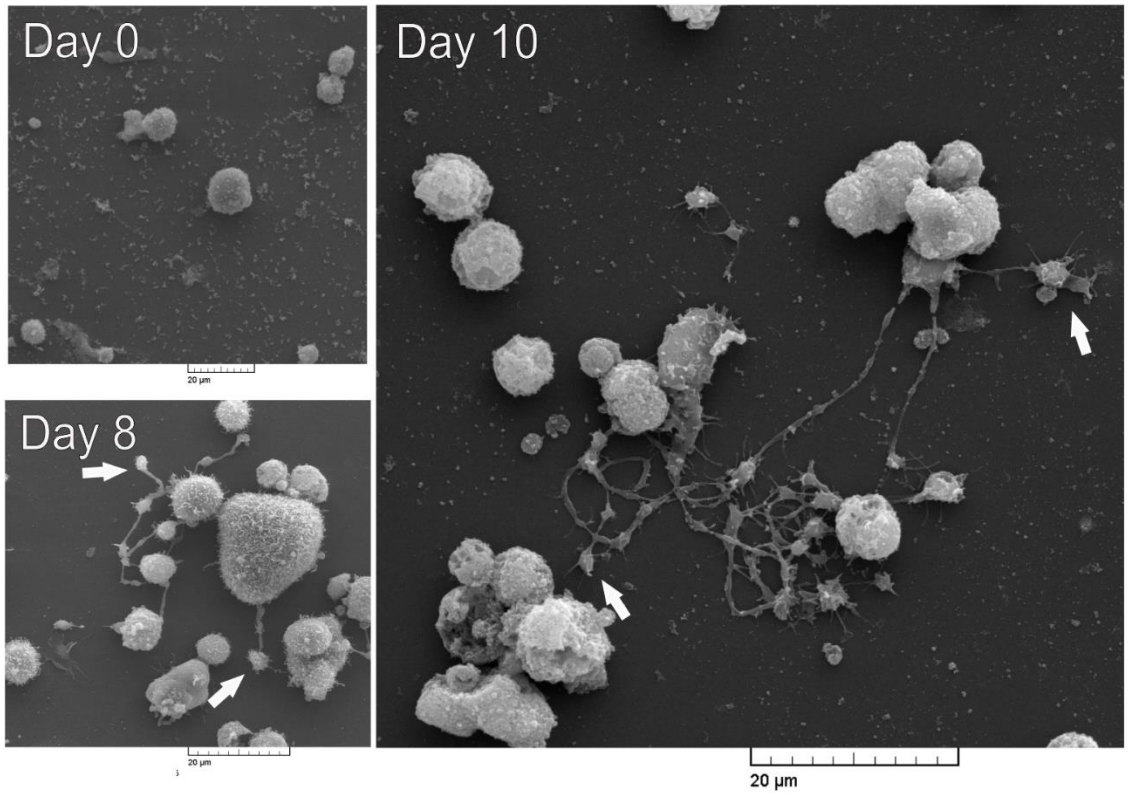

Figure 2.5: Cultured megakaryocytes form proplatelet extensions
Expanded HSPCs undergoing megakaryopoiesis were transferred to fibrinogen coated glass slides on days 0, 8, and 10 and analyzed by scanning electron microscopy. Initially expanded HSPCs (day 0) were identified as small and spherical with no evidence of proplatelet extensions. By days 8 and 10, the cultured cells have increased in size and have begun to form extensive proplatelet formations indicating potential for platelet production (white arrows). Magnification: 2000 X for day 0, 3000 X for days 8 and 10.

2.5 Discussion

In this report, we described the use of a simple two-step culture assay to produce megakaryocytes from the peripheral blood of healthy volunteers. The megakaryocytes were confirmed using electron microscopy and immunophenotyping via flow cytometry. This assay could be used to study normal human megakaryocyte biology, and the impact

of diseases characterized by impaired platelet production.[25,26] Expansion provides a potential source of HSPCs that can be used to test cellular mechanisms in a purely autologous system and can also be applied directly to patient cells to evaluate inherent or transient defects in megakaryopoiesis.

Using the approach others have described, we found that HSPCs isolations yield 1.5 x 10^3 HSPCs per ml of blood donated. This indicates that a 90 ml blood draw would provide 1.4 x10^5 HSPCs, which in turn would seed only two 1 ml well cultures.[13] Therefore, we evaluated the use of an expansion supplement. This increased the initial HSPC population by nine-fold without the loss of viability or CD34 expression. This yield is comparable to one unit of umbilical cord blood which is estimated to possess 1 million HSPCs.[11] Following expansion, we demonstrated that these expanded cells were responsive to TPO stimulation and most committed to megakaryopoiesis as indicated by CD41a and subsequent CD42b expression (83% and 50% of the total cell population, respectively). Overall optimal megakaryocyte growth was determined to post-expansion with TPO concentrations ≥10 ng/ml. As indicated by the electron microscopy pictures (Figure 2.4 and 2.5) these expanded cells were capable of developing into large polyploidy cells that possessed extensive granulation and a demarcated membrane system; morphology associated with mature megakaryocytes.[24] These cells also can form proplatelet extensions in a manner similar to what occurs physiologically; indicating these mature megakaryocytes can likely produce platelets.[1] When comparing expanded and non-expanded HSPC capacity to undergo megakaryopoiesis, preliminary data for CD41a and CD42b expression was found to be similar between groups when cultured under

identical conditions (83±5% vs 78±6% for $CD41a^+$ expression and 50±8% vs 50±25% for $CD41a^+$ $CD42b^+$ at 20 ng/ml of TPO). However, the yield of non-expanded HSPCs was too low to do a complete TPO dose or culture timeline for full comparisons to be made. When comparing results with previous publications using non-expanded peripheral blood derived megakaryocytes grown in similar conditions we found comparable megakaryocyte morphology (by electron microscopy) but lower levels of DNA polyploidy (≥ 4N: expanded HSPC 31%, Non-expanded 62%).[16,27] These results suggest that expansion may have an impact on nuclear polyploidy during megakaryocyte development.[16,27]

In comparison to previous peripheral blood megakaryopoiesis assays, our expansion method requires the lowest amount of peripheral blood while still maintaining a high level of HSPC purity without requiring the need for apheresis. To obtain similar HSPCs yields from peripheral blood using a non-expanded culture would require a blood draw over 800 ml. Furthermore, factoring in the 2.9 fold increase in megakaryocyte growth ($CD41a^+$) after the 9.2 fold increase in HSPCs expansion means that the overall yield is about 27 megakaryocytes for every initial HSPC, which is a significant improvement over other techniques.[8,13,16] This assay uses standard lab culture conditions and a simple TPO driven process to grow mature megakaryocytes. If need be, these results could be further improved by the addition of other supportive cytokines known to increase megakaryocyte yield along with the optimization of megakaryocyte specific culture conditions to provide the ideal growth environment.[28,29]

Expanding peripheral blood derived HSPCs provides a practical venue for growing megakaryocytes and studying megakaryopoiesis and potentially thrombopoiesis. This facilitates direct studies of the megakaryopoiesis process using cells from patient populations with impaired platelet production that range from congenital thrombocytopenia to essential thrombocythemia. [5,30] Peripheral blood derived HSPCs from patients with inherited platelet disorders such as Bernard–Soulier syndrome, MYH9 related disorders, or Quebec Platelet Disorder have been previously used to study megakaryopoiesis; indicating that these patients (and likely other platelet disorders) possess sufficient amounts of HSPCs that can undergo megakaryopoiesis to study the impact mutated or abnormal platelet protein levels have on this vital cell process.[7,13,15,18] By using expansion as a tool, a larger and broader range of patient populations and platelet disorders can to be studied, leading to new insights into homeostatic platelet production and disorder mechanisms.

A peripheral blood assay can also be used to study drug interactions within target populations and make it possible to study cellular interactions between megakaryocytes and other bone marrow cells, including immune cells, in an entirely autologous setting, allowing for better characterization of megakaryocyte cellular interactions within specific patient populations. We believe this is the first report of expanding peripheral blood derived HSPCs and inducing them to undergo megakaryopoiesis resulting in a high yield of mature and functioning megakaryocytes, thus potentiating future studies into the physiology and pathophysiology of megakaryopoiesis.

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2.7 Contribution:

I designed and performed all cell culture experiments and did all flow analysis with assistance from Nadia Karim and Rumi Clare. James Smith and Jane Moore provided technical advice. Marcia Reid from the Faculty of Health Science's Electron Microscopy Facility helped develop the processed samples for imaging. I analyzed the data and prepared the figures and wrote the manuscript. Dr. I. Nazy, Dr. K. Hope, D.M. Arnold, Dr. J.G. Kelton helped with editing.

Table 2.1: Isolation and expansion of CD34+HSPCS from peripheral blood.

	Initial Isolation of HSPCS			**Expansion of HSPCS**		
Isolation	Purity	Viability	HSPCS/ml of blood	CD34%	Viability	Fold Increase*
1	37%	62%	700	80%	87%	8.3
2	85%	83%	1,800	90%	98%	11.2
3	75%	82%	600	86%	92%	9.6
4	98%	90%	2,000	97%	87%	12.2
5	93%	80%	1,400	96%	87%	6.7
6	75%	83%	500	84%	88%	7.7
7	96%	94%	2,500	90%	95%	10.4
8	86%	84%	2,800	90%	88%	14.4
9	88%	78%	500	80%	95%	11.4
10	77%	58%	2,100	94%	96%	5
11	90%	80%	1,600	94%	98%	4
Mean ±SD	82±17%	79±11%	1,500±800	89±6%	92±5%	9.2±3.2

Summary of 11 separate expansion experiments, Results are summarized as the mean ± standard deviation. Overall CD34 expression and viability was maintained during expansion while the absolute $CD34^+$ population increased 9.2-fold.
*Fold increase is calculated as the absolute expanded $CD34^+$ population divided by the initial seeding $CD34^+$ population.

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Chapter 3

Investigating monoclonal antiplatelet antibodies for inhibition of megakaryopoiesis

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3.1 Author's preface

This chapter is focused on testing various monoclonal anti-platelet antibodies to identify any that inhibit megakaryopoiesis. The identification of inhibiting monoclonal antibodies serves multiple purposes, including confirmation that antibody mediated inhibition of megakaryopoiesis is present, the use of these antibodies as models to investigate the mechanisms of inhibition and the establishment of positive controls for the megakaryopoiesis assay developed in Chapter 2. To achieve these goals, I tested anti-GPIIb/IIIa and anti-GPIb antibodies in the megakaryopoiesis assay. To generate enough of the inhibiting monoclonal antibodies for further investigation, hybridoma cell cultures were used to generate more of the monoclonals after which the antibodies were purified, and quality control tested. From the anti-GPIb antibodies, I identified an anti-GPIb antibody that inhibits megakaryocyte maturation, but not megakaryocyte production. This effect was present with the Fab antibody fragment suggesting internal signalling must be involved for causing this effect. From the anti-GPIIb/IIIa antibodies, I identified an antibody that consistently caused megakaryocyte agglutination, rather than inhibition, and that this effect is an *in-vitro* artifact that can be mistaken for inhibition. A. Huynh, J.W. Smith, and H. Bhakta performed enzyme immunoassays and serotonin release assays. J.W. Smith, and A. Huynh performed SDS-PAGE and radioactive immunoprecipitation quality control testing. All other work including experimental design, cell cultures, data analysis and manuscript writing was done by myself. Editing and review of the manuscript were performed by Dr. I. Nazy, Dr. D.M. Arnold, Dr. J.G. Kelton.

3.2 Abstract

Background: Antibody mediated inhibition of megakaryopoiesis may be an important cause of thrombocytopenia in primary or secondary immune thrombocytopenia (ITP); however, this biological mechanism has not been adequately characterized. Control antibodies that inhibit megakaryopoiesis are needed for investigations into the mechanism causing inhibition and to function as screens to identify similar autoantibodies in ITP plasma.

Study Design and Methods: Using a peripheral blood based megakaryopoiesis assay that I developed as part of my thesis, I screened monoclonal antibodies against major platelet/megakaryocyte antigens (GPIIb/III and GPIb) for inhibition of megakaryopoiesis. Inhibiting antibodies were digested and the fragments tested to identify what antibody fragments are responsible for causing inhibition.

Results: Two monoclonal antibodies, an anti-GPIb (TW-1) and an anti-GPIIb/IIIa (Raj-1) affected megakaryopoiesis. TW-1 inhibited maturation of megakaryocytes by 45±14% and this inhibition was retained when testing the Fab antibody fragment, reducing megakaryocyte maturation by 38±8%. Raj-1's effect was determined to be agglutination of megakaryocytes as it was dependent on having both binding sites present on the antibody. This agglutination was present without any indication of cellular activation as p-selectin and PF4 secretion levels were comparable to controls.

Conclusion: Two monoclonal antibodies have been identified for affecting megakaryopoiesis. An anti-GPIb monoclonal antibody that inhibits megakaryocyte maturation and an anti-GPIIb/IIIa that causes megakaryocyte agglutination.

3.3 Introduction

Immune thrombocytopenia (ITP) is a heterogeneous auto-immune blood disorder that reduces circulating platelet levels and can have multiple pathogenic mechanisms.[1,2] The loss of these platelets affects hemostasis and as a result, ITP patients can develop bleeding symptoms ranging from mild bruising and bleeding to intracranial hemorrhaging.[3] Investigations into ITP pathology have uncovered the presence of anti-platelet autoantibodies in some patients which target platelet receptors and cause platelet clearance through the reticuloendothelial system.[4,5] The two most prevalent of these autoantibodies target the platelet receptors glycoprotein(GP) IIb/IIIa and GP-Ib/IX.[6,7] This mechanism of platelet destruction is suspected to be the primary cause of thrombocytopenia in ITP; however, most patients do not have detectable auto-antibodies in their plasma which suggests that additional mechanisms are also occurring.[6,8]

A combination of clinical and research studies show that inhibition of platelet production within the bone marrow may be one of these additional mechanisms. Platelet kinetic studies have demonstrated that up to a third of ITP patients have reduced platelet production levels while being thrombocytopenic.[9,10] Analysis of ITP bone marrow samples also demonstrate that some ITP patients have these anti-platelet autoantibodies sequestrated within the bone marrow compartment.[11] As megakaryocytes are the precursor cells responsible for platelet production, they express the same platelet receptors that are targeted by ITP autoantibodies that cause platelet destruction.[12,13] Using *in-vitro* cell culture assays, plasma and IgG fractions isolated from ITP patients have been

reported to inhibit megakaryopoiesis and their ability to produce platelets.[14–18] This inhibition has been detected without any presence of complement factors nor additional immune cells, indicating a separate mechanism from the traditional antibody effector functions.[16,19]

Although inhibition of megakaryopoiesis by ITP autoantibodies have been reported, the mechanism causing this effect remains unknown. To date, neither the specific identities of these inhibiting autoantibodies nor the significance of their presence in ITP patients and whether this is connected with patient platelet counts, treatment responses or severity of symptoms is known.[15,16,19,20] The difficulties in establishing such connections are likely a result of the difficulties in defining the inhibiting antibodies and separating them from the non-specific or irrelevant antibodies that are detected.[21] Several observations also need to be resolved which contrast with reported inhibition, such as bone marrow samples showing most ITP patients contain megakaryocyte levels comparable to healthy donors, the success of thrombopoietin (TPO) mimetics as a treatment option for ITP patients, and the inability of murine models to replicate this inhibition of megakaryopoiesis *in-vivo*.[22–24] TPO is the key cytokine responsible for inducing platelet production, and the success of TPO mimetics such as romiplostim and eltrombopag, which have been demonstrated to increase platelet counts in over 80% of ITP patients, suggest thrombocytopenia can be alleviated by increasing platelet production, but does not explain how TPO could overcome an autoantibody mediated effect.[25–27]

One limitation of current research is the lack of well characterized monoclonal antibodies which can be used to investigate the mechanisms involved in megakaryopoiesis inhibition and can function as a reference to identify patient samples with similar inhibiting autoantibodies. To address these issues, I report the initial screening of several anti-GPIIb/IIIa and anti-GPIb monoclonal antibodies for their influence on megakaryopoiesis. From this screen, I identified two inhibiting antibodies: an anti-GPIIb/IIIa known as Raj-1, and an anti-GPIb known as TW-1. Further study of these antibodies determined that the effect caused by Raj-1 was due to agglutination whereas TW-1 inhibition affects megakaryocyte maturation and is likely caused by internal receptor signalling pathway.

3.4 Methods

Isolation of HSPC from peripheral blood

Hematopoietic stem and progenitor cells (HSPC) were purified, cultured and expanded as previously published.[28] This process included collecting peripheral blood from healthy donors into sterile commercial BD acid citrate dextrose (ACD) solution B glass tubes (cat 364816). The platelet rich plasma was removed, and the peripheral blood mononuclear cells were isolated using density centrifugation spins on Histopaque (cat 1077, Sigma-Aldrich) and then on a prepared Optiprep density of 1.063 g/ml to remove any remaining platelets. (Optiprep, Axis-Shield, Oslo Norway).[29,30] The resulting cell pellet was washed and the HSPCs ($CD34^+$) were isolated by using the StemSep human $CD34^+$ positive selection kit (cat 18056, StemCell Technologies, Vancouver, Canada) via

a magnetic separation column, as per manufacturer's instructions (cat 130-090-976 QuadroMac Separator, cat 130-042-401 Multistand, cat 130-042-401 Separation Columns, cat 130-041-407 Pre-separation filters all from Miltenyi Biotec, Bergisch Gladbach, Germany). Once isolated, HSPC were analyzed for $CD34^+$ and $CD45^+$ expression using flow cytometry, while viability and the absolute cell count was determined using a TC-20 automatic hemocytometer (cat 145-0102 Bio-Rad, Hercules, USA). This study was completed with approval from the Hamilton Integrated Research Ethics Board, with all samples being obtained with informed, written consent.

Expansion of HSPCs

The expansion of HSPCs was previously published: isolated HSPCs were suspended in culture media containing Iscove's Modified Dulbecco's Medium consisted of GlutaMax (cat 31980030 Life Technologies Carlsbad USA), BIT-9500 serum substitute (15% v/v, cat 09500 StemCell Technologies), 100 units/ml of penicillin, and 100 µg/ml of streptomycin.[28] The cells were suspended to a final concentration of $5.0x10^4$ HSPCs ($CD34^+$) per ml prior to expansion. Isolated HSPCs were expanded using the StemSpan $CD34^+$ Expansion cocktail (cat 02691 StemCell Technologies), with the supplement being mixed in a 10% (v/v) ratio with cultured media. The cells were then expanded for 4 days at 37°C and 5% CO_2. The expansion purity and viability were analyzed using the same methods as the initial isolation.

Megakaryopoiesis Assay

The expanded HSPC were washed and suspended in fresh culture media (5.0×10^4 $CD34^+$ cells /ml) before being placed in triplicate 200 µL wells inside a 96-well plate. This media was the same composition as the one used during expansion, except the expansion supplement was replaced with recombinant TPO at concentrations of either 6 ng/ml or 20 ng/ml and 50 ng/ml of stem cell factor (SCF). (cat 288-TP & cat 255-SC-010/CF R&D Systems, Minneapolis, USA). The addition of TPO was defined as day 0 of the megakaryopoiesis culture. Megakaryocyte growth and maturation status were evaluated by measuring the expression of CD41a and CD42b (See flow cytometry analysis). The absolute number of megakaryocytes was determined as either the product of the live cell count as measured by the TC-20 automatic hemocytometer (cat 145-0102 Bio-Rad, Hercules, USA) and the percentage of cells which expressed the relevant marker as determined by flow cytometry or through the direct use of a volumetric cell counter.

Testing for inhibition of megakaryopoiesis by monoclonal antibodies

Monoclonal antibodies (10 µg/ml) were added to expanded stem cells at the start of TPO stimulation in a 10% (v/v) ratio set in triplicate wells (200 µL of 50,000 CD34+ cells/ml) for 8 days at a TPO concentration of 6 ng/ml, unless otherwise specified. To determine what effect the monoclonal antibodies or their fragments had on mature megakaryocytes, the antibodies were added to mature megakaryocytes cultured with a saturating (20 ng/ml) TPO concentration on day 8 (10% v/v) by which time cultured megakaryocytes would be expected to be fully mature. Inhibitors Cytochalasin B

(Cayman Chemical Company cat 11328), Src-I1 (Sigma-Aldrich, Cat S2075) and PP2 (Sigma-Aldrich, Cat P0042) were incubated before monoclonal treatment to determine their potential effect on attenuating inhibition of megakaryopoiesis.

Flow Cytometry Analysis

All antibodies and flow cytometry stains were obtained from BD Biosciences (Franklin Lakes, USA). Isolated HSPCs were labelled with fluorescein isothiocyanate (FITC) conjugated CD45 monoclonal antibody (Cat 555482) and phycoerythrin-cyano 5 (PECy5) conjugated CD34 monoclonal antibody (Cat 555823). HSPCs are identified as $CD45^+$ $CD34^+$ and purity was calculated as the percentage of $CD34^+$ events within the $CD45^+$ gate.[31] Following expansion, cells were assessed for CD34 expression using the same CD34-PECy5 monoclonal antibody. Appropriately matched isotype controls were used to establish background fluorescence for subsequent identification.

CD41a-FITC (cat 555466), CD42b-Phycoerythrin (PE, cat 555473), CD62P-Allophycocyanin (APC, Cat 550888) and properly matched isotypes were used to identify megakaryocytes ($CD41a^+$), their maturation status ($CD41a/CD42b^+$) and p-selectin expression ($CD62P^+$). Cells were analyzed with the CytoFLEX flow cytometer using Immunon 1B plates (100-µl aliquots of each replicate), and wells were stained directly for 30 minutes at room temperature in the dark with the labelled antibodies. 7AAD staining (cat 988981E) was then performed for 10 minutes at room temperature to identify dead cells and exclude them from subsequent analysis. Samples were diluted to a final volume of 200 µl with PBS 1% BSA and analyzed directly on the flow cytometer. Platelets were

identified by their small forward size and side scatter properties, as either $CD41a^+$ or $CD42b^+$ cells where appropriate. Forward scatter signal width and forward scatter signal height were used to identify singlets.

Large megakaryocyte clusters were identified as $CD41a^+$ events that were not singlets based off forward scatter signal width and forward scatter signal height measurements. A medium control was used as a negative control reference to identify these clusters and concentrations were measured using the volumetric function of the Cytoflex Flow Cytometer.

Antibody purification and digestion

Raj-1 and TW-1 antibodies from cultured hybridoma cell supernatant or ascites fluid were purified using affinity chromatography. Purification was performed by using protein G beads to bind murine IgG antibodies from cultured supernatant before elution using an acid solution of 0.1 M Glycine (pH 2.8). The buffer was then neutralized and exchanged into PBS using a concentrator. The purified monoclonal antibody was tested to confirm specificity using radioimmunoprecipitation, and the purity of the antibody is assessed using Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Purity was determined to be greater than 90%, and reactivity of TW-1 and Raj-1 was confirmed to be specific to glycoproteins Ib and IIb/IIIa. Antibody concentrations were determined using O.D. reading at 280 nm wavelength with a spectrophotometer and filter sterilized using a 0.2 µm filter. All other commercial antibodies were bought and processed identically to the purified IgG. Catalogue numbers for screened monoclonal antibodies were as follows:

HIP1 (Thermal fisher Cat 14-0429-80), HIP2 (Thermal fisher Cat MA119382), M148 (Abcam Cat ab11024), AK2 (Life Technologies cat MA516565).

Antibody fragments were generated using the commercially available digestion kits from Pierce Thermofisher. For Fab and F(ab)'2 generation, a ficin preparation kit (Cat 44980) and a pepsin preparation kit (Cat 44688) was used, respectively. Digestion was followed as per the kit manufacturer's protocol.

Culturing Hybridomas

Cultured hybridomas of Raj-1 or TW-1 were grown in media composed of H-cell 100 Serum (10% v/v), (Cat 001035), RPMI, glutamine and penicillin/streptomycin in T-75 flasks. Cells were cultured to confluency and then incubated for an additional 14 days. Once cultured, cells were removed by centrifugation (700 g, 15 minutes) and the supernatant was purified using a protein G column as described in methods.

PF4 measurement and secretion assay for megakaryocyte activation

To detect megakaryocyte activation, the secretion of platelet factor 4 (PF4) was measured using an enzyme immunoassay. Cultured megakaryocytes ($2x10^6$ cells/ml) were incubated in 200 µl aliquots for 30 minutes with the following conditions: Raj-1 (5µg/ml), Thrombin (1 U/ml), negative control murine IgG (5µg/ml). Triton X-100 (1% v/v) and Raj-1 preincubated with IV.3 were also tested once as controls. Following incubation, the cells were centrifuged (300 g, 15 minutes) and the supernatant was collected and measured for the presence of PF4 using a modified PF4/heparin IgG-

specific EIA. 96-well NUNC Maxisorp plates (Thermo Fisher Scientific cat 44-2404-21) were pre-coated with 10 µg/ml of streptavidin and 1 IU/ml biotinylated-heparin and blocked with PBS with 3% BSA for 2 hours at ambient temperature. 100 µl of cultured supernatant was added and incubated for 1 hour at ambient temperature. 0.1 µg/ml rabbit polyclonal anti-PF4 (LifeSpan Biosciences Inc. cat LS-C662670-100) was added to wells and incubated for 1 hour at ambient temperature. After washing, alkaline phosphatase-conjugated anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA) was added at a 1:5000 dilution and incubated for 1 hour at ambient temperature. Addition of 1 mg/ml p-nitrophenylphosphate (PNPP, Sigma-Aldrich) substrate dissolved in 1 mol/L diethanolamine buffer (pH 9.6) was added for detection. Following a 30-minute incubation, the optical density was measured at 405nm (OD_{405nm}) using a TECAN Sunrise plate reader (Tecan Group Ltd., Mannedorf, Switzerland). Polyclonal rabbit anti-PF4 was used to control and detect the amount of PF4 bound to the heparin in each well. A PF4 standard curve was collected for each analysis and all supernatants were tested in replicates.

Serotonin release assay (SRA) for platelet activation

The ability of the purified antibodies and their fragments to activate platelets was measured using the serotonin release assay. The functional SRA was performed as previously published by Kelton et al.[32] Platelet-rich plasma was prepared by differential centrifugation of whole blood (250 x g 10 minutes) collected using acid-citrate dextrose (ACD). 11 pre-screened donors, who were known to react well to pathogenic HIT

antibodies, were used. Platelets were radiolabeled with ^{14}C-serotonin and washed twice in calcium/albumin-free Tyrode's solution (CAF). Cells were resuspended in albumin-free Tyrode's buffer (AFT) (75 µL, 350,000/µL) and incubated 60 minutes at 22ºC with test antibodies. Following the addition of phosphate buffered saline with 5 mM ethylenediaminetetraacetic acid (PBS-EDTA) and centrifugation, 50 µL of supernatant was removed and counted in a scintillation counter (Packard Bell, Topcount) to measure the release of ^{14}C-serotonin. The amount of serotonin release in each well was compared to the total radioactivity in 75 µl of the platelet preparation and expressed as a percentage of the total amount used. The SRA result was considered positive for activation *if* the sample caused ≥20% serotonin release.[3]

Confocal microscopy of cultured megakaryocytes

Sterile cell chamber slides were coated with poly-lysine (Sigma, 25488-63-0) at a concentration of 0.01% for 5 minutes and allowed to air dry for 30 minutes. Mature megakaryocytes were then added to the chambers slides at a concentration of 150,000 cells/ml (1 ml of culture to 2 cm^2 surface) and left for incubation overnight. After incubation, media was exchanged with 300 µl of commercial Human AB sera as a block for 1 hour at 36ºC. Afterwards, the sera were removed and 300 µL of test antibody (Raj-1 or M148) was added at a concentration of 5 µg/ml then incubated for 40 minutes. The chambers were washed with 300 µL of PBS before the addition of 300 µL of 4% PFA fixative (Sigma F8775). After 30 minutes, slides were washed 3x with PBS and stored at 4ºC until staining. Staining was performed by incubating cells with 3% BSA and 0.2%

Triton X-100 for 1 hour. Samples were then washed two times with 500 µl of PBS before the addition of a goat anti-mouse secondary Alexa 488 (2 µg/ml in 3% BSA) (Abcam, cat A11001) and incubated for 3 hours. The solution was then drawn off and washed before the addition of 200 µl of Flash Phalloidin™ Red 594 (1:25 dilution from stock) (Biolegend, cat 424203) for 20 minutes. The chambers were then washed 5x with 500 µl of PBS before the addition of DAPI stained mounting media and sealed with acetone with a coverslip. Following this, the slides were stored until they were analyzed on the EVO widefield microscope.

Dynamic light scattering

Antibody fragments were screened for aggregation using autocorrelation analysis of quasielastically scattered light in a technique known as dynamic light scattering. Autocorrelation functions were collected from 50 µl of approx. 0.5 mg/ml protein samples after a 2-minute equilibration period for an average of 15 reads (10 seconds per read) at 37°C. Sample intensities were then converted into size distributions using the Stokes-Einstein equation. All samples were tested using a Zetasizer Nano ZS and in duplicate to confirm results.

Statistical analysis

All data representing the number of positive CD41a and CD42b cells are presented as mean ± standard deviation and are normalized to a background medium control for comparisons across experiments. Absolute cell counts are represented as the

mean ± standard deviation for any single experiment. All data was analyzed using Excel or GraphPad Prism 6 and all flow cytometry data was analyzed by Flowjo version 10.4. Results were considered statistically significant if the calculated *p*-value was less than 0.05 in comparison to the relevant negative control.

3.5 Results

Influence of anti-GPIb monoclonal antibodies on megakaryocyte growth

Three monoclonal anti-GPIb antibodies (AK2, HIP1, TW-1) were screened for inhibition of megakaryopoiesis, and only TW-1 was found to inhibit megakaryocyte maturation (Figure 3.1). As the CD42b flow antibody could be blocked by some of the monoclonal antibodies tested, megakaryopoiesis was quantified by the number of $CD41a^{+}$ cells cultured, and their maturation status by the mean fluorescence intensity (MFI) of CD41a expression, which increases as megakaryocytes mature. Of the three monoclonal antibodies tested, only TW-1 reduced the MFI for CD41a expression, indicating inhibition of megakaryocyte maturation (Figure 3.1). Repeated testing showed that TW-1 significantly reduced the MFI of CD41a+ expression to 55±14% relative to medium baseline while a negative antibody control had no affect at (108±17%, student T-test p= 0.02, Figure 3.1). TW-1 was tested in a dose response to confirm inhibition was dose dependent on the mature megakaryocyte population (Figure 3.1). Relative to medium control, TW-1 reduced the CD41a+ cell population to 79±5%, and the CD41a mean fluorescence intensity to 53±3% at concentrations of 1 µg/ml or higher. The negative

control murine IgG did not show any inhibition at 10 µg/ml (108±2%, 104±4 for each parameter respectively).

TW-1 also reduced the number of mature megakaryocytes grown ($CD41a^+$ $CD42b^+$) by approximately 50%, however the antibody partially blocked the detecting CD42b antibody probe causing a drop of 10% in MFI when testing with surrogate platelets. The addition of IV.3 (a FcRγIIa receptor blocking antibody) had no effect on attenuating TW-1 inhibition of megakaryocyte maturation (data not shown).

Effect of TW-1 on mature megakaryocytes and thrombopoiesis

TW-1 was added to mature megakaryocytes and caused an immediate increase in the number of cell clusters within 40 minutes of incubation. As shown in Figure 3.2, TW-1 caused a two-fold increase in the number of cell clusters or clumps whereas the negative control antibody had no effect. However, the number of $CD41a^+$ cells decreased by only 8±5% when treated with TW-1 suggesting that the number of cells within the cluster is small. TW-1 is not known to activate platelets and this was confirmed for megakaryocytes as well since p-selectin expression based on MFI was comparable to the negative murine control antibody (Figure 3.2).[33] Thrombin as a positive control caused a 235±42% increase in p-selectin expression as measured by MFI.

To determine its influence on platelet release, TW-1 was added to mature megakaryocytes and incubated for either 40 minutes or overnight and the number of platelets present was then measured. It was determined that a 40-minute incubation decreased platelet levels by 6±10% whereas overnight incubation reduced it by 20±2% in

comparison to the negative control antibody tested. This effect could be attributed to either agglutination of megakaryocytes or inhibition of thrombopoiesis and further work will be necessary to differentiate between the two possibilities.

TW-1 Fab inhibits megakaryocyte maturation

To determine whether the TW-1 effect on megakaryocytes is a result of agglutination, the antibody was digested into the Fab fragment and tested for causing inhibition. This fragment was generated after a 3-hour digest period using papain with purity confirmed using a non-reduced SDS-PAGE (Figure 3.3). To detect any protein aggregation, the TW-1 Fab fragment was analyzed using dynamic light scattering. As shown in Figure 3.4 most of the protein sample (99%) had the same size and diameter as the reference (Raj-1) Fab fragment with a measured diameter of ~6 nm. A small presence of protein aggregate was detected (0.2%) with a diameter of ~90 nm. This amount is considered too small to be of significance.

The TW-1 Fab fragment inhibited the development of the mature megakaryocyte population with levels comparable to full length TW-1 (Figure 3.5). After 8 days of culture at 5 µg/ml, the TW-1 Fab fragment reduced CD41a expression on cells with MFI dropping to 60% in relation to the medium control. This reduction was similar to the full-length TW-1 antibody effect (55%), whereas the negative control antibody had no impact (113%). When testing the TW-1 Fab fragment, there was little change in the number of $CD41a^+$ cells cultured, but a large reduction in the mature megakaryocyte population was observed with mature megakaryocyte ($CD41a^+CD42b^+$) counts reduced to 67% of the

medium control. Unlike the full-length TW-1, the smaller Fab antibody fragment did not block the CD42b detection flow antibody, indicating this effect is biological rather than some form of blocking interference. These results are the average of two separate experiments conducted in experimental triplicates.

When comparing the TW-1 Fab fragment to medium control in three separate experiments, the mature megakaryocyte cell population was reduced to 62±8% of the medium control with a reduction in CD41a fluorescence to 61±7%; this inhibition was statistically significant using the paired student t-test ($p<0.02$, paired T-test).

Adding monoclonal TW-1 Fab fragment to megakaryocytes results in cell death

To determine whether TW-1 Fab fragment inhibits megakaryocyte growth or causes megakaryocyte death, the fragment was added to a mature megakaryocyte culture (day 6) and analyzed two days afterwards (Figure 3.6). The live mature megakaryocyte cell count ($CD41a^{+}CD42b^{+}$) decreased by 47±15% when referenced against the medium control and was 55±13% of the initial megakaryocyte population observed on day 6. These observations also correspond to a decrease in the viability of the mature megakaryocyte population (59±11%) in comparison to the medium control (75±2%). A decrease in CD41a MFI was also observed with levels declining to 68±4%.

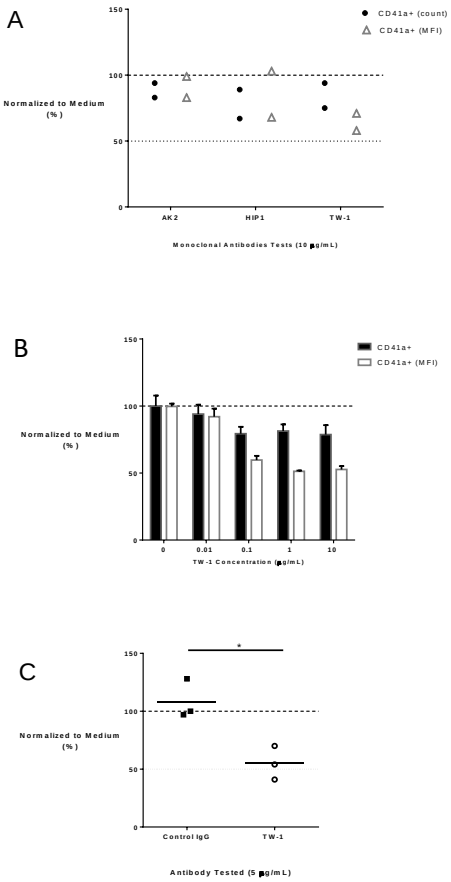

Figure 3.1: Screening anti-GPIb antibodies for effect on megakaryopoiesis.
(A) Three monoclonal anti-GPIb antibodies were screened for inhibition in the megakaryopoiesis assay for 8 days at 10 µg/ml. TW-1 reduced the MFI of CD41a (Δ) to 64% indicating an inhibitory effect. Each antibody was tested in two separate experiments with a representative Figure showing the results of the average of each experiment.
(B) TW-1 was tested in a titration to determine dose dependence. Relative to the medium control, TW-1 reduced the CD41a+ cell population to 79±5% and the CD41a mean fluorescence intensity to 53±3% at concentrations of 1 µg/ml or higher. The negative control murine IgG did not show any inhibition at 10 µg/ml (108±2%, 107±2% and 104±4 for each parameter respectively). Results are the summary of a single triplicate experiment represented as the mean± standard deviation.
(C) Repeated testing of the TW-1 antibody in comparison to a non-specific murine antibody of the same antibody class and subgroup as TW-1 indicated the reduction in $CD41a^{+}$ MFI was specific, reproducible, and statistically significant (Paired T-test P=0.02). Each dot represents the average of an experiment with a set of triplicates. Antibodies were tested at a concentration of 5 µg/ml.

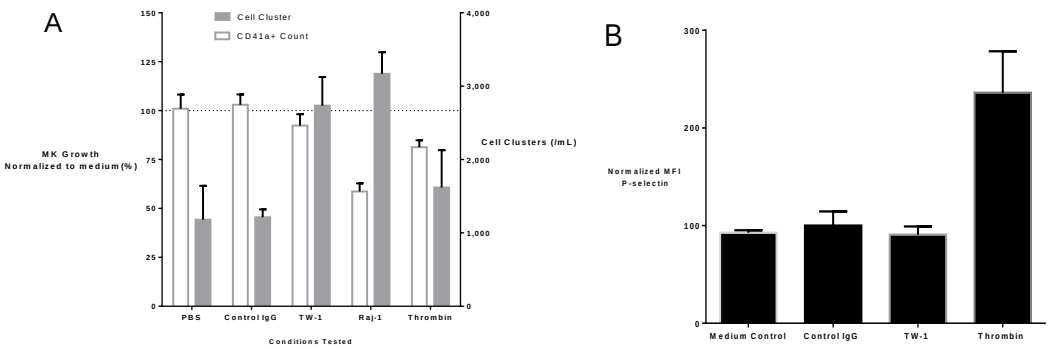

Figure 3.2: Full-length TW-1 causes minor agglutination with no activation
TW-1 was added to mature megakaryocytes at 5 µg/ml and incubated for 40 minutes before the analysis of its immediate effect on cells. Control IgG and Raj-1 were used as negative and positive controls for agglutination. (A) A loss in the absolute number of single cell megakaryocytes ($CD41a^+$) was observed with numbers dropping by 8±5% and 52±7% for TW-1 and Raj-1 respectively in relation to medium control (white bars left axis). When counting the number of cell clumps both Raj-1 and TW-1 caused a 2-fold increase in comparison to medium and negative antibody control (grey bars right axis). The discrepancy between the number of clumps and reduction in megakaryocyte counts is likely a factor of the size of the aggregates. Both Raj-1 and TW-1 caused more clustering than thrombin (1 U/ml). (B) P-selectin expression based on MFI levels was measured for TW-1 and was found to be comparable to the negative control, indicating no megakaryocyte activation was occurring. This Figure is a representative of one of two separate experiments conducted in triplicates (mean ± standard deviation).

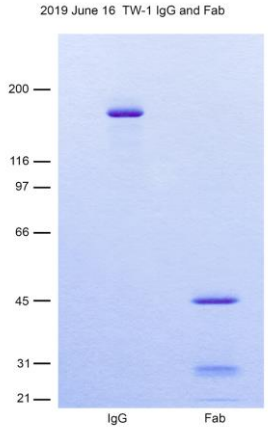

Figure 3.3: Non-reducing SDS PAGE gel outlining the digested fragments of TW-1
A non-reducing SDS-PAGE gel was run with the digested fragments of TW-1. A protein ladder is listed on the side with corresponding molecular weights labelled. Full length (fl) purified TW-1 was ~150 kDa in size while the Fab fragment was ~45 kDa.

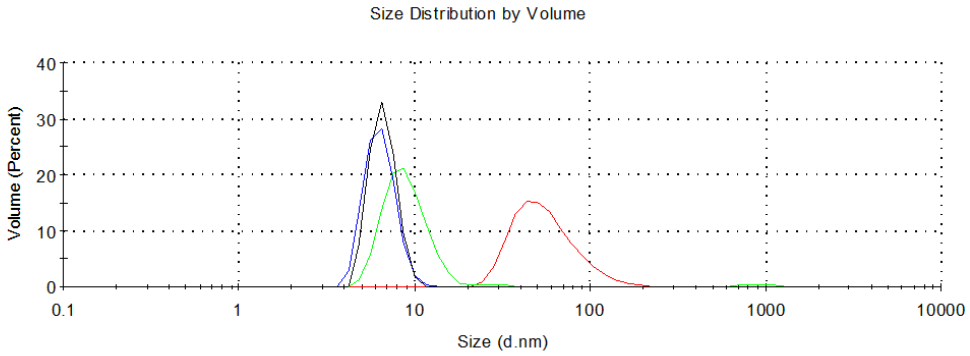

Figure 3.4: Dynamic light scattering shows no significant aggregation of TW-1 Fab

The TW-1 Fab fragment (Blue) was screened using dynamic light scattering for protein aggregates. For controls: full length TW-1 (green), reference Fab (black) and heat aggregate IgG (red) were also screened. As shown by the size distribution overall most of the TW-1 Fab fragment aligns with the Fab fragment reference with both samples reporting a diameter of ~6 nm. Full length TW-1 was measured to be at 12 nm and control heat aggregated IgG was 86 nm. An aggregate peak was detected for TW-1 Fab with a diameter of ~90 nm however it was estimated to constitute less than 0.2% of the total protein amount. All samples were tested at 37 degrees Celsius to copy culture conditions and all fragments were thawed and left overnight at 37 degrees to allow any aggregates to form.

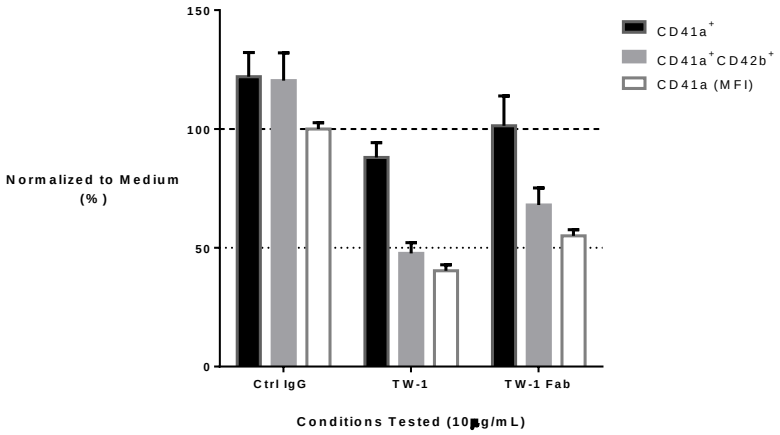

Figure 3.5: TW-1 Fab inhibits the mature megakaryopoiesis population
The TW-1 Fab fragment was added to HSPC as they were stimulated to undergo megakaryopoiesis and revealed to inhibit the growth of the mature megakaryocyte population with levels comparable to full length TW-1 (all conditions were tested at 5 µg/ml). The TW-1 Fab fragment lowered the mature megakaryocyte population to 68±7% of the medium control and dropped CD41a MFI to 56±2%, whereas the negative IgG control did not (120±12% and 100±3%). All numbers were normalized to the medium control and represented as the mean with standard deviation. This Figure is a representative plot of one of two separate experiments conducted in triplicate with similar results tested.

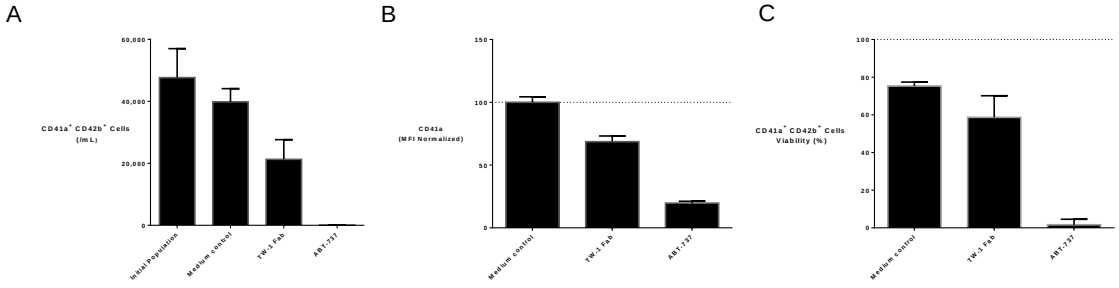

Figure 3.6: Adding monoclonal TW-1 Fab fragment to mature megakaryocytes results in cell death
To determine whether the effects of TW-1 Fab are inhibiting megakaryocyte growth or causing mature megakaryocyte death, the TW-1 Fab fragment (5 µg/ml) was added to a megakaryocyte culture on day six and analyzed two days later. (A) The live mature megakaryocyte population dropped to 47±15% of medium control and 55±13% of the starting population on day 6. (B) These observations matched a drop in the viability of the mature megakaryocyte population as well as a decrease in the MFI for CD41a expression (normalized to medium). ABT-737 was used as a positive control to induce cell death and caused similar effects. These results are from a single experiment represented as the mean ± standard deviation.

Influence of anti-GP IIb/IIIa monoclonal antibodies on megakaryopoiesis

The influence of three monoclonal murine anti-GPIIb/IIIa antibodies (Raj-1, M148, HIP2) on megakaryocyte growth are summarized in Figure 3.7. Raj-1 reduced the $CD42b^+$ cell population by 75±25% in comparison to the medium control while the two other antibodies had no effect. This inhibition was also present when measuring CD41a cell counts and it was confirmed that the antibody did not interfere with the CD41a detecting antibody used for flow cytometry measurements (data not shown). Raj-1 binding to cultured megakaryocytes was confirmed by confocal microscopy (Figure 3.9).

Repeated testing of Raj-1 demonstrates that its effect was consistent, specific, and statistically significant as testing at 5 µg/ml reduced megakaryocyte growth (CD41a) to 40±7% of medium control while a non-specific negative antibody control of the same antibody class and subgroup was 108±16% (T-test, p=0.002, Figure 3.7). Inhibition was dose dependent and plateaued at 10 µg/ml with megakaryocyte growth at 18±3% of the medium control (Figure 3.7). As Raj-1 is known to activate platelets through the FcRγIIa receptor, the addition of IV.3 was added in increasing concentrations and this was found to have no effect on attenuating Raj-1 inhibition (data not shown).

Effect of Raj-1 on mature megakaryocytes and platelets

To determine the immediate influence of Raj-1 on mature megakaryocytes, the antibody was added to mature megakaryocytes and incubated for 40 minutes before analysis. Raj-1 caused a rapid reduction in the number of megakaryocytes within the $CD41a^+$ cell count, decreasing it to 41±17% of medium reference while in comparison the

negative control IgG had no effect (100±7%) (Figure 3.8). This Raj-1 decrease was dose dependent, having a saturating effect at concentrations greater than 1 µg/ml (Figure 3.8). Forward signal scatter and side scatter analysis demonstrated the presence of large cellular clusters positive for $CD41a^{+}$, which were counted using forward scatter signal width and height measurements (Figure 3.8). The number of these megakaryocyte clusters had a peak concentration at 1 µg/ml before beginning to decrease in count.

The addition of Raj-1 also caused an immediate reduction in the number of cultured platelets present in the assay with levels decreasing to 42±1% and plateauing at 1 µg/ml in tandem with the observed reduction in megakaryocyte counts (data not shown). Similar observations were also noted when testing platelets treated with IV.3 indicating possible agglutination.

Raj-1's effect is not a result of activation

The addition of Raj-1 to developed megakaryocytes induced an immediate presence of large cell clusters. As Raj-1 is known to activate platelets and megakaryocyte can be activated by agonists such as thrombin, this suggested that the antibody was either activating cells or causing agglutination.[33,34] Following Raj-1 incubation with mature megakaryocytes, p-selectin expression was measured on mature ($CD42b^{+}$) megakaryocytes. Based on MFI, p-selectin was found to be comparable to the negative control antibody and medium control (75±8% for Raj-1 and 109±5% for antibody control in comparison to medium reference); indicating that Raj-1 does not induce activation (Figure 3.10). Thrombin as a positive control doubled the level of p-selectin expression

(216±9%). When counting the number of large cell clusters, thrombin caused only a slight increase in cell clustering (137±40%) as oppose to Raj-1 (269±25%) when normalized to the medium control. The negative control antibody had no effect (103±10%). Titrating Raj-1 to lower concentrations did not affect p-selectin expression, which remained comparable to medium control (data not shown). Experiments were repeated twice to confirm the results.

These findings were supported using an enzyme-linked immunosorbent assay to detect activation by measuring the secretion of platelet factor 4 (PF4). PF4 is an α-granule protein endogenously produced in megakaryocytes and secreted during platelet activation.[35] After incubation with Raj-1, the supernatant of the megakaryocyte culture had PF4 levels comparable to negative antibody medium controls (97% vs 105%, numbers normalized to medium control and are represented as the average of two experiments, table 3.1). Thrombin was used as a positive control and induced PF4 secretion doubling the amount of detectable PF4 by 200% in comparison to the reference medium (table 3.1). Triton was also tested once and was found to release a similar amount of PF4 as thrombin (401 ng/ml vs 309 ng/ml, Table 3.1). These results support the conclusion that Raj-1 causes agglutination and not activation following its addition to cultured megakaryocytes.

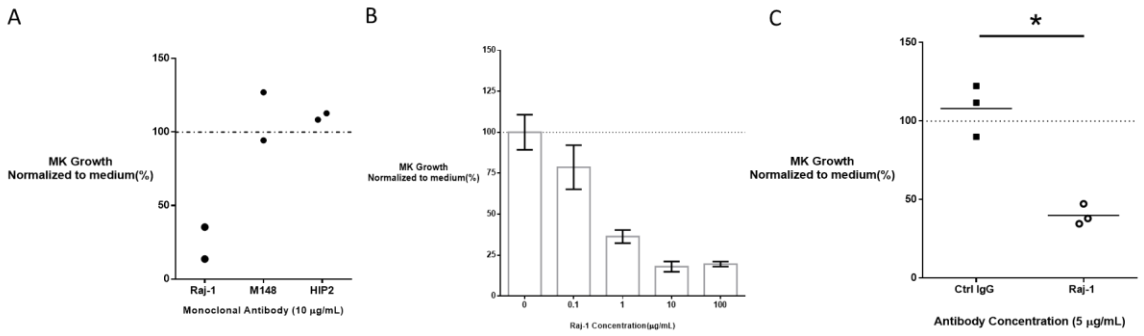

Figure 3.7: Raj-1 has an inhibitory, dose dependent and specific effect on megakaryopoiesis

(A) Monoclonal anti-GPIIb/IIIa antibodies were added to expanded HSPC undergoing megakaryopoiesis. After 8 days of culture, the number of mature megakaryocytes ($CD42b^+$) was counted and normalized to a medium control. Raj-1 reduced megakaryocyte growth ($CD42b^+$ cells) by 75% in comparison to the medium control (dotted line). Samples were tested at a final concentration of 10 µg/ml. Results are the summary of two separate experiments each represented by a single dot as the average of three replicates. Megakaryopoiesis was measuring using CD42b expression to avoid any steric interference with the CD41a detecting flow antibody. Raj-1 was verified to not block the detecting CD41a antibody probe thereby allowing CD41a+ analysis for subsequent experiments.

(B) Raj-1 inhibition was dose dependent as increasing antibody concentrations resulted in lower megakaryocyte growth ($CD41a^+$) with a plateau occurring at 10 µg/ml with growth at 18±3% when normalized to the medium control. Results are the summary of a single experiment with triplicates and represented as the mean ± standard deviation.

(C) Raj-1 was incubated at 5 µg/ml with expanded HSPC undergoing megakaryopoiesis for 8 days with a midpoint TPO concentration of 6 ng/ml. In comparison to a nonspecific murine antibody control of the same antibody class and group (Ctrl), Raj-1 significantly reduced megakaryocyte growth ($CD41a^+$) to 40±6% of medium control while the control antibody had no effect (108±16%). Each dot represents a separate experiment conducted in triplicates with the mean represented by the line. Inhibition was statistically significant and specific (paired T-test, $P<0.05$).

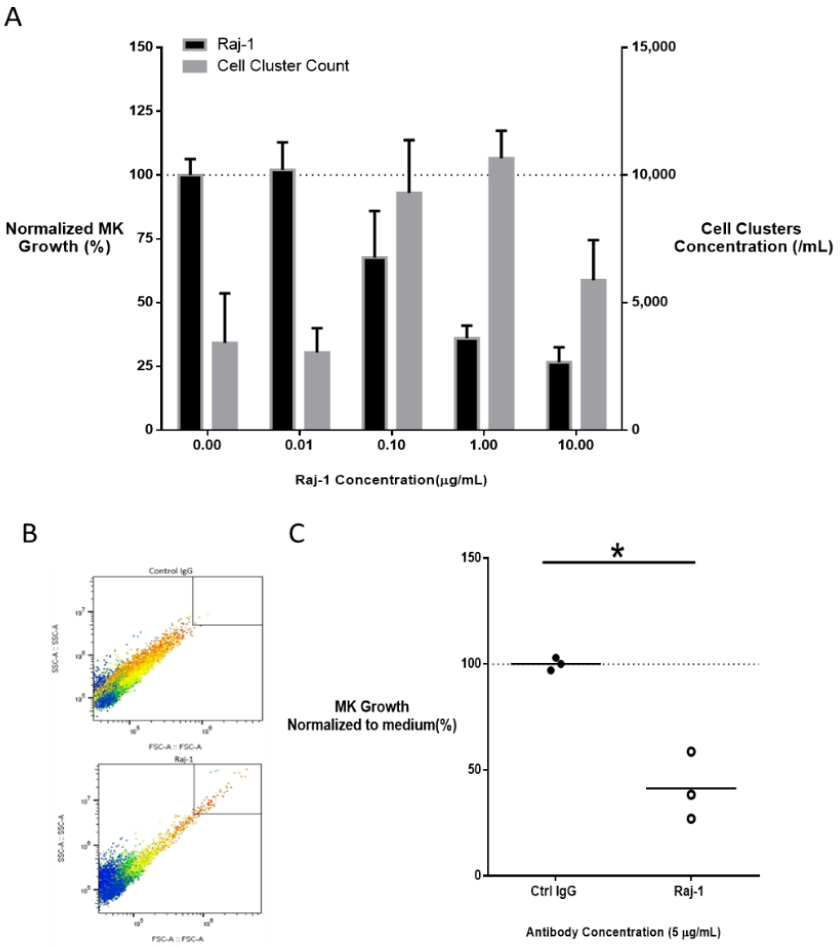

Figure 3.8: Raj-1 induces immediate cell clustering following 40 minutes of incubation with mature megakaryocytes.

(A) Increasing Raj-1 concentrations caused a decrease in the number of megakaryocytes ($CD41a^+$) with a saturating effect detected at a concentration greater than 1 µg/ml (results are normalized to medium control and represented as black bars to the left axis). An increase in the number of large cell clusters (grey bar, right axis) corresponded with the reduction in megakaryocyte numbers with a peak cell cluster concentration detected at 1 µg/ml. (B) Forward and side scatter analysis of the cultured megakaryocytes showed an aggregation of cells following Raj-1 incubation as highlighted by the upper right gate. A heat map representing CD41a expression levels from low (blue) to high (orange) is imposed to demonstrate the aggregate cell clusters contain megakaryocytes. (C) This immediate effect caused by Raj-1 was reproducible and identical to previous inhibition results reported for the 8-day culture periods reported (41±17% vs 40±6%). Each dot represents a separate experiment conducted in triplicates with the mean represented by the line. Results were statistically significant in comparison to the negative control (Ctrl) antibody (paired T-test, $P<0.05$).

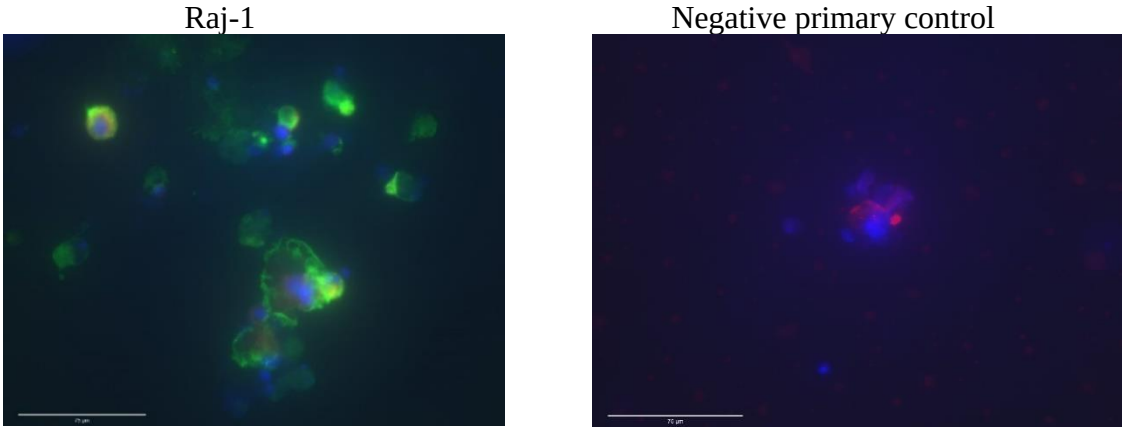

Figure 3.9: Confocal microscopy images of Raj-1
Mature megakaryocytes were transferred to polyline coated slides and labelled with either Raj-1 or a non-specific murine antibody of the same subclass and group. Cells were stained for DNA (DAPI-Blue), actin (Phalloidin-RED) and a secondary anti-mouse goat antibody (Green) before being analyzed on an EVOS FL 2 widefield microscope. The objective was to confirm specific Raj-1 binding to cultured megakaryocytes. The white bar on the lower left portion of the graph represents a scale of 75-microns in length.

Raj-1's causes agglutination and is independent of internal signalling

As Raj-1 did not cause activation, we investigated whether the reduction in megakaryocyte numbers could be explained by megakaryocyte agglutination. To confirm this possibility, Raj-1 was digested into its Fab and F(ab)'2 fragments using ficin and pepsin digest kits and these fragments were tested in the megakaryopoiesis assay. As the Fab fragment would only have one antigen binding site it would be unable to form aggregates unlike the F(ab)'2 and full-length protein. As shown in Figure 3.11, only the full-length antibody and F(ab)'2 fragments inhibited megakaryocyte growth by reducing it to 35±6% and 41±4% of the medium control, while the Fab and negative control IgG were 89±15% and 90±17%. Results were repeated in two separate experiments with the

same observations noticed. The F(ab)'2 and Fab fragments did not activate platelets as serotonin release was not detected (data not shown). This meant that the effect of Raj-1 was dependent on having both antibody binding sites present on the antibody (bivalent binding). To support this result, Fab was added in 10-fold excess to mature megakaryocytes before the addition of full-length Raj-1 in a blocking assay. After a 40-minute incubation, the number of megakaryocytes was 71±20% in the Fab blocked samples, whereas it was 38±3% in the Raj-1 samples when referenced against the medium control.

Clustering of GP IIb/IIIa receptors on the platelet surface is known to cause internal signalling through the "outside in" signalling pathway and promote activation of platelets.[36] To confirm this is not the case with Raj-1 on megakaryocytes, we blocked this signalling pathway using the inhibitors PP2 and Src-I1. These inhibitors stop the Src family of kinases from signalling, which are the dominant series of effectors activated from "outside in" GP IIb/IIIa signalling.[37–39] As shown in Figure 3.12, the inhibitors failed to attenuate Raj-1 mediated agglutination. To confirm inhibitor function, they were tested in the serotonin release assay, the most sensitive platelet activation assay, in which they inhibited platelet activation by Raj-1 at the tested inhibitor concentration. In addition, we also tested Cytochalasin B, which is a potent inhibitor of platelet aggregation by halting actin polymerization, to determine if it can reduce the observed level of agglutination caused by Raj-1 on megakaryocytes. As shown in Figure 3.13, the addition of the inhibitor alleviated 50% of the effect caused by Raj-1, however, the inhibition was not completely attenuated indicating that the Raj-1 effect is still present even when the

motility of the cell is inhibited. Together these results demonstrate that the Raj-1 effect is agglutination caused by the bivalent binding of the antibody and is independent of internal signalling processes. This means that the inhibition detected by Raj-1 on megakaryopoiesis is a result of an *in-vitro* culture assay.

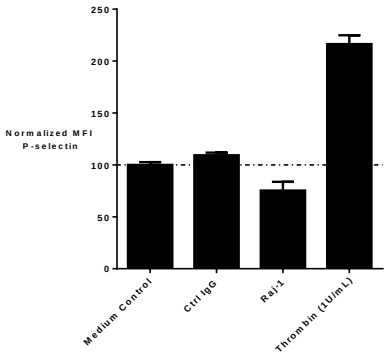

Figure 3.10: Raj-1 does not induce p-selectin expression.
Raj-1 (5µg/ml), Thrombin (1 U/ml) or negative antibody control were added to mature megakaryocytes and incubated for 40 minutes before analysis for p-selectin expression. P-selectin was measured as the MFI of the mature megakaryocyte population ($CD42b^+$) using the CD62P-APC probe, numbers were normalized to a medium control and the Figure is a representative of one of two experiments performed with the mean and standard deviation of 3 experimental triplicates.

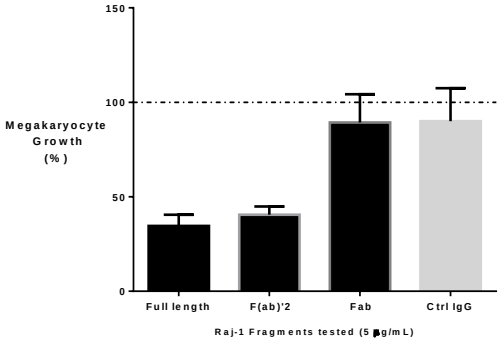

Figure 3.11: Raj-1s effect on megakaryocytes is dependent on having both binding sites
Purified Raj-1 and its fragments were screened for inhibition in the megakaryopoiesis assay at a concentration of 5 µg/ml. After 8 days of cell culture, only the full length and F(ab)'2 fragments influenced megakaryocyte ($CD41a^+$) growth; reducing it to 35±6% and 41±4% of medium baseline while the Fab and negative control IgG were 76±19% and 84±7%. A representative Figure of one of two experiments performed is shown with the mean and standard deviation error bars.

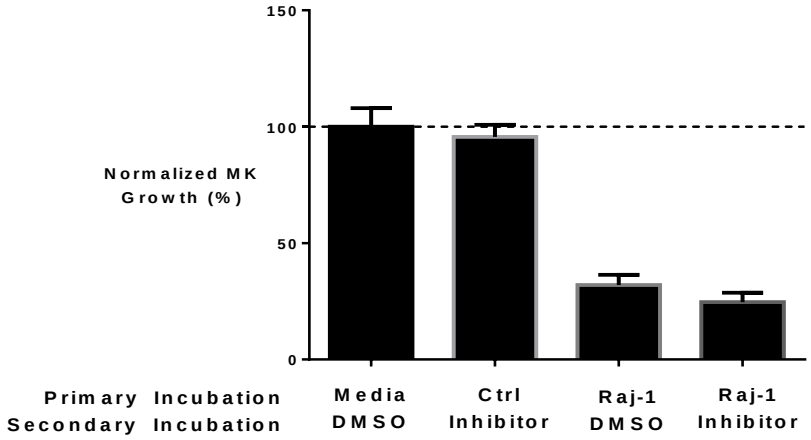

Figure 3.12: Inhibitors of SRK signalling do not affect agglutination caused by Raj-1
To determine whether Raj-1 induces "outside in" signalling similar to platelets, an inhibitor cocktail comprising of PP2 and Src-I1 was preincubated for 1 hour with mature megakaryocytes (Concentration 2 mM each). Test antibodies were then added and incubated for 40 minutes before cells were analyzed on flow cytometry to measure the $CD41a^{+}$ megakaryocyte cell count (normalized to medium control). The inhibitors had no impact on Raj-1's agglutination. DMSO (<1% v/v) was used as a control vehicle for the media and Raj-1 samples as the inhibitors were stored in such a solution. Results are the summary of one experiment performed in triplicates.

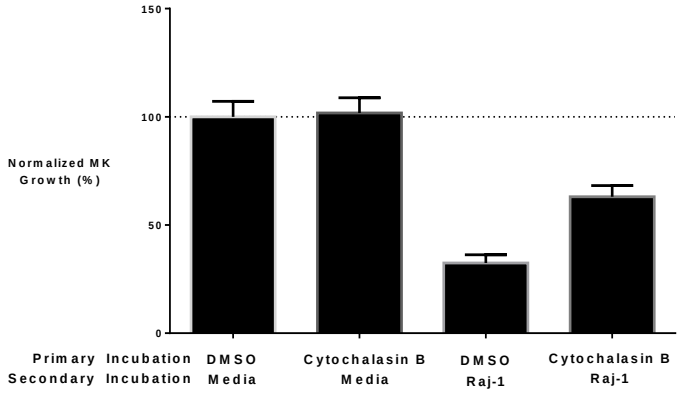

Figure 3.13: Cytochalasin B halts some agglutination caused by Raj-1
The actin inhibitor Cytochalasin B was added to mature megakaryocytes at a concentration of 50 µM for 1 hour before the addition of Raj-1 (5 µg/ml). Following a 40-minute incubation period, the number of live CD41a+ megakaryocytes was analyzed and normalized to medium control. Since Cytochalasin is stored in DMSO, buffer controls containing DMSO (<1% v/v) were used. The addition of the inhibitor stopped 50% of the agglutination caused by Raj-1, however, the effect was still present even with the cell's motility inhibited. Results are the summary of one experiment performed in triplicates

3.6 Discussion

Immune thrombocytopenia is a heterogeneous autoimmune blood disorder with evidence of impaired platelet production occurring in a subgroup of patients.[15,16,19,40] Several publications have shown that ITP plasma positive for anti-GPIIb/IIIa or anti-GPIb autoantibodies can inhibit megakaryopoiesis and thrombopoiesis, however, the mechanism causing this effect is not understood.[14,16,41] A challenge to this research is the lack of monoclonal antibodies that can be used as models to investigate antibody inhibition of megakaryopoiesis. These antibodies can serve as proof of concept demonstrating this inhibition and as positive controls for future experiments to investigate the mechanisms responsible for causing this effect. As a result, we tested and identified two monoclonal antibodies that affect megakaryopoiesis; one was determined to inhibit megakaryocyte maturation while the other causes agglutination.

Testing anti-GPIb monoclonal antibodies for influence on megakaryopoiesis demonstrated that TW-1 inhibits megakaryocyte maturation and reduces the mature megakaryocyte population ($CD41a^+$ $CD42b^+$). This effect of TW-1 was retained when testing the Fab antibody fragment, which indicates downstream signalling from the GPIb receptor is likely responsible for causing this inhibition.

The GPIb receptor is known to be important for megakaryocyte development and has been associated with apoptotic regulation in platelets. Genetic alterations of the cytoplasmic region of the GPIb protein results in impaired megakaryocyte development

in mouse models.[42,43] Those cultured mutants exhibited underdevelopment of the demarcated membrane and a reduction in proliferation potential; all of which could interfere with the cell's capacity to produce platelets. [42,43] From those studies, the GPIb receptor is shown to be capable of regulating signalling proteins such as 14-3-3ζ, and in turn, could affect the PI3K/AKT and by extension, TPO signalling pathways.[42] On platelets, the GPIb receptor is part of the GPIb/IX/V signalling complex and interacts with multiple different signalling pathways including 14-3-3ζ and PI3K which are known regulators of apoptosis. [20,44–47] Clustering of the GPIb receptor on cold treated platelets is known to induce apoptosis.[46] Since TW-1 is not known to cause activation nor aggregation, this suggests that perhaps the antibody could be interfering with the formation of the GPIb/IX/V signalling complex, unbalancing regulatory signalling that could affect megakaryocyte development. [48] This could explain why the antibody has no noticeable effect on platelets but impacts megakaryocytes.

Impaired platelet production from an anti-GPIb autoantibody, in our studies, does align with some observations published in the literature for ITP pathology. It has been observed that ITP patients with anti-GPIb autoantibodies are more resistant to conventional IVIG treatment. Furthermore, ITP plasma positive for anti-GPIb antibodies had stronger associations with impaired megakaryocyte growth in previous megakaryopoiesis studies.[49–52] Another monoclonal anti-GPIb known as SZ2 has been reported to also cause platelet apoptosis via AKT signalling through the GPIb/IX receptor which has been found to inhibit megakaryocyte growth in colony-forming assays.[53,54]

Anti-GPIb antibodies from other disorders, such as drug induced thrombocytopenia, have also been demonstrated to inhibit megakaryopoiesis and proplatelet formation.[53,55]

Testing anti-GPIIb/IIIa monoclonal antibodies for inhibition of megakaryopoiesis revealed the presence of agglutinating antibodies such as Raj-1. Following platelet activation, the main function of GPIIb/IIIa is to undergo a conformational change and bind to other GPIIb/IIIa receptors by using fibrinogen as an intermediate crosslinking protein. [56,57] This results in platelet aggregation and the formation of a plug to seal damaged blood vessels.[56,57] It appears that Raj-1 binds to GPIIb/IIIa in a specific orientation such that it mimetics this crosslinking and causes cellular agglutination. Unlike regular GPIIb/IIIa activation, this type of binding would bypass any requirement for initial activation and would not generate any "outside in" signalling from GPIIb/IIIa.[58] This outcome would explain why the Src inhibitors have no effect on Raj-1 mediated agglutination and why there is no p-selectin expression or PF4 secretion.[37,38,59] Cytochalasin B partially alleviated Raj-1 agglutination indicating the actin cytoskeleton supports the adhesion. However, because the attenuation was only partial it would also indicate that the effect is independent of any cell motility.[60,61] Since aggregation was present only when both antibody binding sites remain intact, it is clear Raj-1 causes agglutination and this precedes any other effect observed with the cultured megakaryocytes. This agglutination would likely result in cell death within the cell clusters, as access to nutrition is restricted. This type of inhibition is unlikely to be present *in vivo* as megakaryocyte clustering is not detected in bone marrow samples and the

overall low frequency of megakaryocytes in the environment (<0.1%) would make such an interaction improbable.[22,62] As a result, this type of "inhibition" is in fact agglutination and so can yield misleading conclusions when it comes to identifying impaired megakaryopoiesis *in-vitro*.

Screening multiple anti-GPIIb/IIIa and anti-GPIb antibodies demonstrate that only a subset of them affect megakaryopoiesis. This variation can be attributed to either different epitopes being bound or having different affinities for their target receptors, but simply binding to the target receptor alone is not enough to cause inhibition and other factors need to be considered. In ITP, autoantibodies are suspected to bind to a few localized regions on the GPIIb/IIIa and GPIb/IX receptors, but it is not known what regions or epitopes are important for megakaryocyte development.[63,64] It is likely that some autoantibodies would bind but have no effect on the megakaryocyte as they are in the wrong epitope or have an insufficient affinity to their targeted receptor. This would explain previous publications that report not every ITP plasma positive for an anti-GPIb or anti-GPIIb/IIIa autoantibody causes inhibition of megakaryopoiesis, as some of these autoantibodies would likely be irrelevant.[15,19]

Further subcategorization of inhibiting antibodies is needed to identify impaired platelet production in ITP patients; specifically identifying if they bind to epitopes shown to inhibit megakaryopoiesis. By identifying TW-1 as an inhibiting antibody, and its effect is not caused by agglutination (as in the case with Raj-1), we can confirm that antibody

mediated inhibition of megakaryopoiesis is possible. Furthermore, TW-1 can now be used as a screening aid to identify ITP patients with similar anti-GPIb autoantibodies. The monoclonal antibody can also be used as a control to investigate the mechanism responsible for causing this effect. This information would aid our understanding of how inhibition of megakaryopoiesis occurs as well as be used to identify ITP patients with potentially impaired platelet production.

In summary, a set of monoclonal antiplatelet antibodies targeting GPIIb/IIIa and GPIb were screened to determine their influence on megakaryopoiesis. Two potential antibodies were identified that could affect platelet production. From the work on Raj-1 we can confirm there is an epitope on GPIIb/IIIa that when bound by an antibody would cause severe agglutination within the megakaryopoiesis assay that appears like inhibition but is not. From the work on TW-1, we can confirm there is an epitope on GPIb that when bound by an antibody can inhibit megakaryocyte maturation. This antibody can now be used to further investigate the mechanism responsible for causing this effect as well as a screen to determine if ITP patients have similar autoantibodies.

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3.8 Contribution:

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

3.9 Disclosure of conflicts of interest

The other authors state that they have no conflict of interests.

	Experiment 1		Experiment 2	
	O.D. 405nm	[PF4] (ng/ml)	O.D. 405nm	[PF4] (ng/ml)
Media	1.14	150	0.354	27
Control IgG	1.1345	147	0.368	31
Raj	0.9865	84	0.395	38
Thrombin	1.5135	309	0.475	56
Triton X-100 1%	1.728	401	n.a.	n.a.

Table 3.1: Raj-1 does not induce PF4 secretion

To investigate whether Raj-1 induces megakaryocyte activation, the secretion of PF4 was measured using an enzyme linked immunosorbent assay (EIA). Cultured megakaryocytes ($2*10^6$ cells/ml) were incubated in 200 µl aliquots for 30 minutes with the following conditions: Raj-1 (5µg/ml), Thrombin (1 U/ml), negative control murine IgG or Triton X-100 (1% v/v). Following incubation, the supernatant was collected and analyzed on an EIA. A PF4 standard curve was used to determine the PF4 concentration based on the optical density reading. Results show that Raj-1 does not induce PF4 secretion as levels are comparable to media or negative murine IgG control rather than the positive thrombin and triton X-100 (1%v/v). This experiment was repeated with the same conclusion.

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Chapter 4

Investigating the effect of ITP plasma on megakaryocyte inhibition

Nikola Ivetic, Ishac Nazi, Rumi Claire, John G. Kelton, Donald M. Arnold

4.1 Author's preface

This chapter is focused on testing plasma from ITP patients to determine its effect on platelet production. This study used HSPC isolated from the ITP patients themselves, making this the first autologous study to investigate the effect ITP plasma has on the patient's own megakaryocytes. I found that the addition of patient plasma did not inhibit megakaryopoiesis, and the number of megakaryocytes cultured was either comparable or higher than healthy controls. A significant reduction in the number of platelets generated (de novo) was detected following the addition of ITP plasma, indicating that inhibition of thrombopoiesis was present. A secondary observation was that a subset of ITP HSPC had an inherently enhanced potential for megakaryopoiesis in comparison to healthy controls and this was present without the addition of plasma and at identical TPO concentrations. In addition, these results confirm the feasibility of using this developed assay to study platelet production using ITP patient HSPC. I also tested a Glanzmann plasma sample positive for polyclonal anti-GPIIb/IIIa antibodies and results were similar to what was found with Chapter 3 and the agglutinating anti-GPIIb/IIIa monoclonal Raj-1. J.W. Smith and A. Huynh performed SDS-PAGE as controls to confirm antibody depletion. All cell cultures work, and flow cytometry analysis was performed by NI. Dr. D.M. Arnold assisted with clinical patient selection and R. Clare and M. St John helped provide clinical information. All data analysis, Figures and manuscript writing were done by N. Ivetic while Dr. I. Nazy, Dr. D.M. Arnold, Dr. J.G. Kelton performed editing.

4.2 Abstract

Background: Studies focused on megakaryocyte number and function are needed to understand platelet underproduction as a potential mechanism of immune thrombocytopenia (ITP). Most experimental studies have relied on animal models or exogenous and non-autologous sources of hematopoietic stem and progenitor cells (HSPCs) as a source of megakaryocytic cells. We used autologous cultured megakaryopoiesis to examine the effect of ITP plasma on platelet underproduction.

Study Design and Methods: Megakaryocytes were produced from peripheral blood $CD34^+$ HSCs directly from patients with ITP and healthy controls. HSPC ($CD45^+$ $CD34^+$) were isolated, expanded for 4 days and then cultured with thrombopoietin (TPO) and stem cell factor (SCF) for 8 days. Megakaryocyte numbers were evaluated by flow cytometry using CD41a expression for megakaryopoiesis and CD42b expression for maturation. Platelet production was analyzed using CD41a expression and platelet forward and side scatter profile sizes. The addition of autologous ITP plasma and plasma depleted of IgG were also tested to determine its effects on culture.

Results: Peripheral blood derived megakaryocytes were cultured from HSPCs from ITP patients (N=10), and normal controls (N=9). Approximately 2,200±1,100 hematopoietic stem and progenitor cells were isolated per ml of peripheral blood and a 5-fold increase in stem cells was obtained after an expansion of the isolated ITP patient HSPC population. Following an additional 8 days of culture with TPO, 67±12% of the cultured cells were CD41a+ indicating they were undergoing megakaryopoiesis and 54±15% of the CD41a+ population expressed the CD42b marker for mature megakaryocytes. Of the 10 ITP

cultures grown, four demonstrated an increased potential to develop mature megakaryocytes in comparison to healthy controls. The addition of ITP plasma did not inhibit megakaryocyte growth or maturity, but platelet production was reduced with two samples demonstrating a 50% reduction in platelet levels in comparison to the lowest healthy control. Relative to the number of mature megakaryocytes cultured there was a lower number of platelets detected in ITP cultures (3.34±1.9 platelets per mature megakaryocyte) in comparison to the healthy control group (8.1±1.4 platelets per mature megakaryocyte).

Conclusions: The addition of patient plasma had no inhibiting effect on megakaryocyte counts in an autologous assay but a significant decrease in platelet number (thrombopoiesis) was detected. A subset of ITP HSPC generated more megakaryocytes when compared to healthy donors, indicating an enhanced potential of these cells to generate megakaryocytes. This is to our knowledge the first autologous investigation of ITP plasma on patient HSPC undergoing megakaryopoiesis.

4.3 Introduction

Immune thrombocytopenia (ITP) is an autoimmune platelet disorder characterized by thrombocytopenia and a variable risk of bleeding.[1] ITP pathology was thought to be a result of accelerated platelet destruction; however, it is clear impaired platelet production is also an important feature.[2] Basic investigations focusing on quantitative and functional alterations of megakaryopoiesis and thrombopoiesis are needed to understand the mechanism of impaired platelet production in ITP.

Platelet production is a complex process in which newly formed platelets are created from mature bone marrow cells called megakaryocytes. These cells are continually regenerated from hematopoietic stem cells and this process, termed as megakaryopoiesis, is regulated by the cytokine thrombopoietin (TPO).[3,4] Stimulation by TPO results in stem cell commitment and differentiation into the megakaryocyte lineage as indicated by the early expression of the platelet receptor glycoprotein (GP) IIb/IIIa (CD41a) and late expression of GP Ib (CD42b).[5] Megakaryocyte progenitors undergo cycles of endomitosis resulting in a polyploid nucleus that is thought to support maturation and the development of platelet-associated organelles.[4] Once mature, megakaryocytes undergo thrombopoiesis where they migrate towards the bone marrow sinusoids and release pseudopod-like proplatelet projections from their membrane, which bud off into newly formed platelets in circulation.[4]

Evidence in support of impaired platelet production in ITP derives from several sources. First, TPO receptor agonists, which stimulate megakaryocyte growth and platelet production, effectively increase the platelet count in over 80% of patients with ITP.[1,6–8] Second, based on in-vivo radiolabelled platelet survival studies, ITP patients have a platelet turnover rate that is lower than expected for their degree of thrombocytopenia.[9] Third, cell culture assays show that ITP autoantibodies or autoreactive immune cells can inhibit megakaryopoiesis and/or thrombopoiesis *in-vitro*. [2,10–14] Taken together, these observations demonstrate platelet production is impaired in some ITP patients and improving platelet production can overcome the thrombocytopenia present. The mechanism behind impaired platelet production is not known.

As megakaryocytes reside in the bone marrow and comprise ~0.03% of the total cell population, they are rare and challenging to access.[15] As a result, most ITP studies have relied on animal models or in-vitro megakaryopoiesis cultures.[13,16–18] These models are limited as they cross species or use rare stem cells harvested from healthy donors as their starting cell culture, making these studies allogenic. [2,10–14,19] Therefore, an autologous and accessible human megakaryopoiesis culture system capable of producing functional megakaryocytes is desirable.

In this study, we investigated the use of a megakaryopoiesis assay using peripheral blood derived hematopoietic stem and progenitor cells (HSPCs) isolated directly from ITP patients. Peripheral blood is an easily accessible yet low yield source of

hematopoietic stem cells; therefore we investigated whether the initial stem cell population can be expanded to better facilitate its use for autologous ITP megakaryopoiesis research.[20] We determined whether hematopoietic stem and progenitor cells (HSPCs) can be isolated from ITP patient peripheral blood, whether they can be expanded to a sufficient quantity to subsequently undergo megakaryopoiesis to develop into mature megakaryocytes. Furthermore, we tested the effect of ITP plasma in this megakaryopoiesis assay to demonstrate its functional effects on these expanded HSPC undergoing megakaryopoiesis. To our knowledge, this expansion system has not been accomplished with patient peripheral blood, and this system could make peripheral blood a useful and important source for future ITP research.[21,22]

4.4 Methods

Patient selection criteria for isolation

Patients were identified from the McMaster ITP Registry. This registry enrolls consecutive adult patients attending a tertiary hematology clinic for thrombocytopenia platelet count (150 x 10^9/L). We selected primary ITP patients who were diagnosed based on established criteria, including isolated thrombocytopenia (<100 x10^9/L) with no other identifiable causes. [23] Specifically, we selected ITP patients with historically low platelets counts when possible. Heat treated plasma samples from 11 ITP patients and six normal controls were tested. Clinical details of the ITP patients are outlined in Table 4.1. A plasma sample positive for polyclonal anti-GPIIb/IIIa antibodies was also collected from a Glanzmann Thrombocythemia patient for study. Study approval was received

from the Hamilton Integrated Ethics Board and informed written consent was obtained from all patients and healthy controls.

Isolation of ITP HSPCs from peripheral blood

Peripheral blood was collected from ITP patients or healthy donors and stored in sterile acid citrate dextrose (ACD) anticoagulant tubes (Beckman Dickinson, cat 02-684-26). The blood was depleted of platelet rich plasma following differential centrifugation (150g, 20 minutes) and removal of the top platelet rich plasma fraction.[24] The remaining blood was diluted two-fold with isolation buffer, comprised of sterile phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic (EDTA) acid, 1 mM theophylline, 0.15 µM aprotinin, and 0.02 mM prostaglandin E1. The diluted blood was then layered on Histopaque (cat 1077, Sigma-Aldrich) at a 2:1 ratio and centrifuged for 30 minutes at 400g. The peripheral blood mononuclear cells (PBMCs) were then collected, diluted five-fold and centrifuged at 370g for 10 minutes as a wash. The washed PBMC cell pellet was suspended in a final volume of 25 ml in isolation buffer before layering on 15 ml of Optiprep density gradient to deplete contaminating platelet levels as previously published; Optiprep density was 1.063 g/ml and the sample was spun at 350g for 15 minutes (Optiprep, Axis-Shield, Oslo Norway).[25,26] The resulting cell pellet was then diluted to 50 ml with isolation wash buffer and centrifuged at 200g for 12 minutes as a wash. HSPCs ($CD34^{+)}$ were then isolated from PBMC using the StemSep human $CD34^{+}$ positive selection kit (StemCell Technologies, cat 14756 Vancouver, Canada) via a magnetic separation column as per the

manufacturer's instructions (cat 130-090-976 QuadroMac Separator, cat 130-042-401 Multistand, cat 130-042-401 Separation Columns, cat 130-041-407 Pre-separation filters, all from Miltenyi Biotec, Bergisch Gladbach, Germany). Once isolated, cells were analyzed for CD34 and CD45 expression using flow cytometry. Viability and the absolute cell count were determined using a TC-20 automatic hemocytometer (cat 145-0102 Bio-Rad, Hercules, USA). Isolations that did not yield enough cells for initial analysis or expansion (<50,000 HSPC in total) or had purity less than 60% were classified as failed isolations and were not expanded.

Expansion of ITP HSPCs

Isolated HSPCs were suspended in culture media consisting of Iscove's Modified Dulbecco's Medium containing GlutaMax (cat 31980030 Life Technologies Carlsbad USA), supplemented with BIT-9500 serum substitute (15% v/v, cat 09500 StemCell Technologies), 100 units/ml of penicillin, and 100 µg/ml of streptomycin. The cells were suspended to a final concentration of $5.0x10^4$ HSPCs ($CD34^+$) per ml before expansion. Isolated HSPCs were expanded using the StemSpan $CD34^+$ Expansion Supplement (cat 02691 StemCell Technologies). The HSPCs were seeded in 1 ml aliquots in culture media with the expansion supplement at a 10% v/v ratio. The cells were expanded for four days at 37°C and 5% CO_2. Cell counts, CD34 expression, and viability were measured. Absolute HSPC counts were calculated as the product of the percentage of live cells expressing CD34 and the total live cell count which was determined via flow cytometry and an automated hemocytometer (TC-20 Bio-Rad).

Culturing megakaryocytes and testing plasma for inhibition of platelet production

Expanded $CD34^+$ cells were washed and suspended in fresh culture media (5.0×10^4 $CD34^+$ cells /ml) before being seeded in triplicate 200 µL wells in a 96-well plate. The fresh culture media was identical to the expansion media apart from replacing the expansion supplement with recombinant TPO (6 ng/ml) and SCF (50 ng/ml) (cat 288-TP and cat 255-SC-010/CF, R&D Systems, Minneapolis, USA). Since TPO is added to expanded HSPCs, and this begins megakaryopoiesis, we defined this time point as day 0 or baseline for subsequent megakaryopoiesis analysis. The isolation and expansion of HSPCs was treated as a separate process. The expanded HSPC were cultured under these conditions for 8 days. Processed plasma samples were added to the culture on day 0 at a 10% volume ratio as the cells were stimulated to undergo megakaryopoiesis. A PBS medium control was used for normalization to determine the effects of plasma across experiments. A pooled normal control plasma consisting of 6 healthy donors was used as a reference for comparing the effects of Glanzmann plasma. Megakaryocyte growth and maturation status were evaluated by measuring the expression of CD41a and CD42b (see flow cytometry analysis below). The absolute number of megakaryocytes was determined using a volumetric reader on the flow cytometer.

Flow cytometry analysis

All antibodies and flow cytometry stains were obtained from BD Biosciences (Franklin Lakes, USA). Isolated HSPCs were labelled with fluorescein isothiocyanate (FITC)

conjugated CD45 monoclonal antibody and phycoerythrin-cyano 5 (PECy5) conjugated CD34 monoclonal antibody. HSPCs were identified as $CD45^+$ $CD34^+$ and purity was calculated as the percentage of $CD34^+$ events within the $CD45^+$ gate.[27] Following expansion, cells were assessed for CD34 expression using the same CD34-PECy5 monoclonal antibody. Appropriately matched isotype controls and concentrations were used to establish background fluorescence for subsequent positive identification. CD41a-FITC and CD42b-Phycoerythrin (PE) monoclonal antibodies and matched isotype controls were used to identify megakaryocytes ($CD41a^+$) and their maturation status ($CD41a^+$ $CD42b^+$). 7-Aminoactinomycin D (7AAD) staining was used to identify dead cells and exclude them from flow analysis.[28] CD62P-Allophycocyanin (APC) were used to measure platelet activation. Platelets were analyzed based on their unique forward and side scatter characteristics and the expression of CD41a. Large megakaryocyte cell clumps were identified using forward scatter signal width and forward scatter signal height to exclude non-singlets. A medium control was used for reference to identify cell clumps. All cell concentrations were determined using the volumetric function of the Cytoflex flow cytometer. For initial time course studies that measured platelet production, the Beckman Coulter Epic XL-MCL was used and platelet counts were enumerated using counting beads (BD cat 335925). All flow cytometry data were collected using either a Beckman Coulter Epic XL-MCL or Cytoflex, with subsequent analysis being performed using FlowJo software (Version 10.2, Ashland, USA).

Plasma processing and testing

ITP plasma samples were collected and processed from the same blood draw used to isolate the HSPC for culture. Repeated plasma samples known to be positive for autoantibodies were retrieved from the ITP registry and tested for inhibition. Those samples were processed identically to the collected samples during isolation. A platelet rich plasma fraction was collected from the blood donated following an initial centrifugation spin (150 g, 20 minutes). This plasma was further centrifuged at 16,000g for 15 minutes to generate platelet poor plasma. The supernatant of this sample was then heat treated at 56*C for 30 minutes and filter sterilized using a 0.2-micron filter. Samples were separated into aliquots and frozen until testing. For plasma depleted of IgG, heat-treated plasma was diluted two-fold in PBS and then spiked with protein G sepharose beads in a 2:1 volume ratio (Sigma cat 20399). The samples were then incubated for 45 minutes at room temperature before the beads were removed using centrifugation (300g, 1 minute). The supernatant of this incubation was defined as plasma depleted of IgG. Antibody depletion using the beads was determined to be >90% after visual confirmation using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A sepharose bead sham was also used as a control to account for sample dilution. All plasma samples were filter sterilized before testing.

Statistical analysis

All data points are presented as the mean ± standard deviation unless otherwise stated. All data analysis and statistical testing was completed in Excel and Graphpad Prism. All experiments were performed in experimental triplicate with the average of each group

plotted. A standard two deviation cut-off was used to identify samples with statistical significance and the Grubs statistical test was used to identify outliers. The Mann-Whitney U test and student T-test were used to statistically compare groups when appropriate.

4.5 Results

Clinical summary of ITP patients

10 patients from the McMaster ITP Registry donated blood from which HSPC were isolated, expanded and stimulated into megakaryopoiesis, while their autologous plasma was added to investigate its effects on the culturing cells. The average age of the sample group was 60 years old and the medium platelet count at the time of draw was 75 * 10^9/L with a range of 5 – 252 * 10^9/L (Table 4.1). All patients were classified as primary ITP. Some patients at the time of blood draw were under the following ITP medications including IVIG, Nplate, Eltrombopaq, Prednisone, Omeprazole, Cellcept, Azathioprine, and Mycophenolate mofetil. Three patients had not received any medication at the time of donation. Three plasma samples from two of the ITP patients were also selected from our ITP sample repository and were tested. These selected plasma samples, referred to as repeat samples, were known to be positive for either anti-GPIIb/IIIa or anti-GPIb autoantibodies. The medication of the patients at the time of sample collection for these repeats is also listed in Table 4.1.

Isolation and expansion of ITP patient donor HSPC

The isolation results of purifying HSPC from ITP patient peripheral blood are summarized in Table 4.2 alongside healthy controls for comparison. From ITP patient blood, the isolation purity was 84±6% and the overall yield was 2,200±1,100 HSPC per ml of blood donated. From nine healthy donors, the average isolation purity was 79±9% and the yield was 1500±900 HSPC per ml of blood donated. Two out of ten ITP isolations yielded significantly higher amounts of HSPC than healthy donors in comparison (Figure 4.1). There was a higher rate of failed isolations in ITP patients (9/19, 47%) in comparison to healthy donor controls (3/12, 23%). Between the successful and failed isolations within the ITP group, that was no significant difference between platelet counts or medication at the time of blood collection. Repeat isolations from donors that initially failed had a failure rate of (1/3, 33%). Analysis of HSPC levels in peripheral blood mononuclear cells showed no difference between ITP patients and healthy controls (data not shown). Two non-immune thrombocytopenic samples were also tested and failed in isolation; both patients were diagnosed with hypersplenism.

The isolated HSPC cells were expanded for four days and analyzed for the overall increase in HSPC numbers and their final purity. Although both healthy donors and ITP patients had identical HSPC cell purities after four days of expansion (92±4%) it was observed that HSPC from ITP patients had a smaller increase in the cell population (5±1 fold) in comparison to healthy donors (7±2 fold). This observation was statistically significant ($p=0.04$, T-test, Figure 4.1).

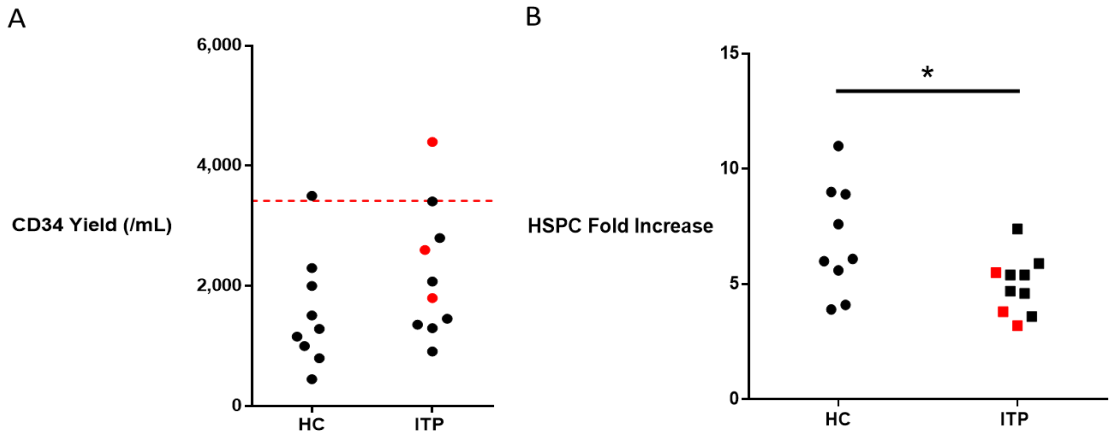

Figure 4.1: ITP samples yielded similar amounts of HSPC in comparison to healthy donors but expanded to a lower amount.
Isolated HSPC from ITP patients or healthy controls (HC) were divided by the volume of blood donated to determine the yield of HSPC per ml of blood. Two out of the ten ITP donors yielded higher amounts of HSPC, as determined using a two-standard deviation cut-off from the HC control population. (T-test, p=0.11). (B) When cultured with expansion supplement for 4 days it was observed that ITP HSPC increased by a smaller margin (5±1 fold) in comparison to healthy donor HSPC (7±2 fold). This result was statistically significant (p=0.04, T-test). Samples highlighted in red were from patients undergoing TPO mimetic treatment at the time of HSPC isolation.

ITP plasma does not affect megakaryocyte growth

To identify any effect ITP plasma may have on megakaryopoiesis and platelet production, autologous heat-treated and filter sterilized plasma was introduced to the expanded HSPC as they were being stimulated with TPO to initiate megakaryopoiesis. Repeated samples positive for anti-platelet autoantibodies in the direct antigen capture assay (>2.0 O.D.) were also selected from the ITP registry and tested autologously. When compared to six healthy donor plasma samples tested, no statistically significant inhibition was detected for either the growth of CD41a+ or CD41a CD42b+ megakaryocytes (Mann-Whitney U test p>0.05), (Figure 4.2). For repeat samples, a

statistical significance was observed only with the mature megakaryocyte population (Mann-Whitney U test p=0.04). Two of the ITP plasma samples increased the CD41a+ cell counts and four increased the mature megakaryocyte population in comparison to the healthy control population. No correlation between megakaryocyte growth or maturation was detected with the patient platelet count at the time of sample collection.

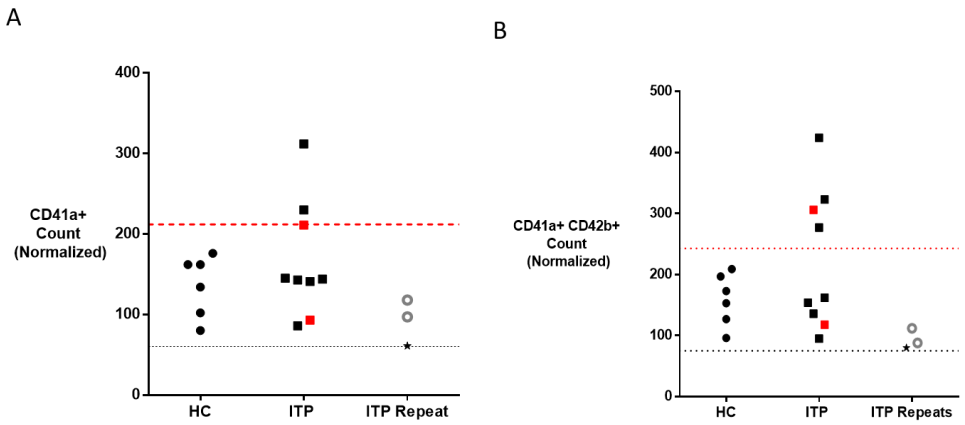

Figure 4.2: No inhibition of megakaryopoiesis was detected from ITP patient plasma When comparing ITP healthy donor (HC) plasma samples for effect on megakaryopoiesis no inhibition was detected, but an increase in both the total number of megakaryocytes (A) and the number of mature megakaryocytes was noticed (B). Megakaryocyte growth (CD41a) was 137±72% in comparison to a medium control while healthy controls were 136±38%. The mature megakaryocyte population was 221±117% for ITP samples and 159±43% for healthy controls. Samples highlighted in red were patients undergoing treatment with TPO mimetics. Three repeat plasma samples were tested that were positive in the direct antigen capture assay; samples with the open circle were positive for anti-GPIIb/IIIa (Optical density >2.0) and the * was positive for anti-GPIb (Optical density of 3.7). Each dot is the mean of a set of triplicates and all counts were normalized to medium control as an internal reference. Red and grey lines represent a two-deviation cut-off from healthy donor experiments. Samples highlighted in red were from patients undergoing TPO mimetic treatment at the time of plasma collection.

ITP plasma does inhibit platelet production

A subset of ITP plasma samples (5/12, including repeat samples) reduced the number of platelets produced de novo in comparison to healthy controls. Three of these ITP samples were below the two standard deviation cut-offs established by healthy donor samples (Figure 4.3). When comparing the ratio of platelets to mature megakaryocytes generated in culture, it was observed that ITP plasma samples had a disproportionately lower ratio than healthy controls (3.34±1.9 vs 8.1±1.4 platelets per mature megakaryocyte). Ten of the twelve plasma samples displayed this lower ratio including repeated plasma samples. This means fewer platelets were produced than expected from the number of megakaryocytes cultured following the addition of ITP plasma in comparison to healthy donors. The healthy donor group had an outlier that produced excessive amounts of platelets in comparison to others as determined by the Grubbs' test. This sample was plotted but removed when establishing cut-offs. A positive linear correlation between the ratio of cultured platelets to mature megakaryocytes and the initial platelet count of the blood sample at donation was detected ($R=0.88$, $P<0.05$). This correlation was insignificant ($R=0.44$, $P=0.12$) when samples with platelet counts above $150*10^9$/L were removed from the analysis (data not shown).

To confirm the detected platelets were cultured and not residual platelets of the initial isolation, a time course of the megakaryopoiesis assay was performed to measure the appearance of platelets. As shown in Figure 4.4, the presence of platelets was minimal during the initial addition of TPO and SCF and begins to increase over time indicating de novo platelet production occurring. It was determined that platelets exponentially decay

in culture every two days to a tenth of the starting population when incubated in media, suggesting a four-day expansion followed by a cell wash and an additional eight-day culture period would eliminate any residual platelet population (data not shown).

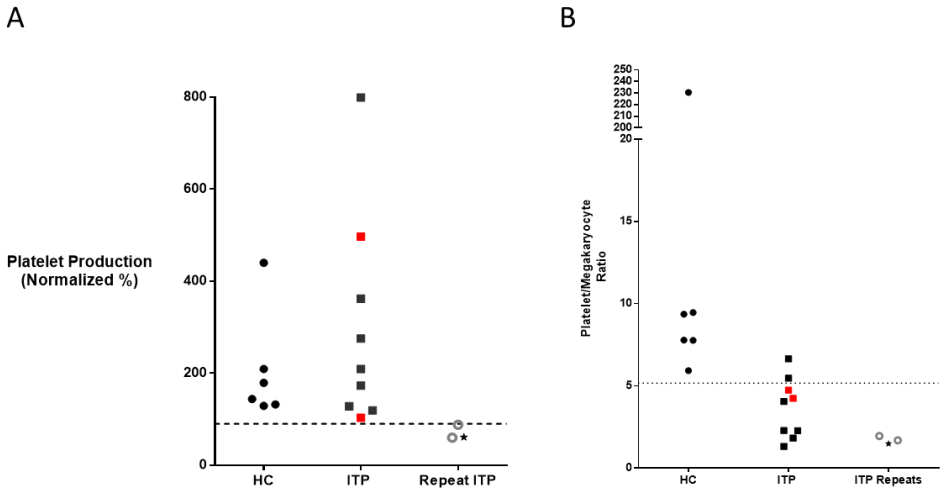

Figure 4.3: A reduction in platelet production was observed when ITP plasma was tested in culture. When comparing ITP plasma to healthy donor samples it was observed that some patient plasmas reduced the number of platelets generated de novo. (A) Three of the twelve ITP samples (including repeats) had a reduction in the number of platelets produced, which was two standard deviations lower than the healthy control (HC) group. (B) When comparing the ratio of platelets produced to the number of mature megakaryocytes cultured, most ITP samples had a lower ratio of platelets to mature megakaryocytes (3.34±1.9 platelets per mature megakaryocyte) in comparison to the HC group (8.1±1.4). The HC had an outlier that exhibited statistically higher platelet production than other samples in the same group and was removed from analysis when calculating the means and cut-offs. Each dot is the mean of a set of triplicates and all counts were normalized to medium control as an internal reference. Samples highlighted in red were from patients undergoing TPO mimetic treatment at the time of isolation

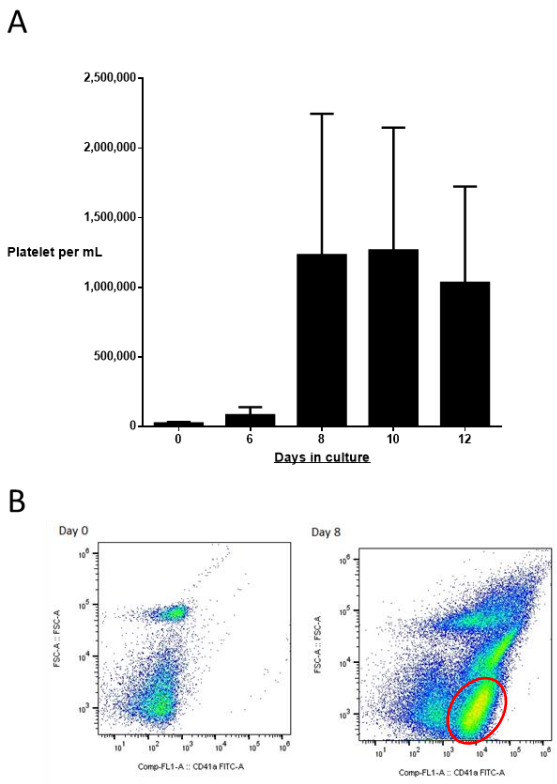

Figure 4.4: The megakaryopoiesis assay produces platelets.
(A) Expanded HSPC treated with a midpoint TPO concentration and SCF. The overall production of platelet sized particles (CD41a+) was measured using counting beads on a Beckman coulter epic-BCL. Results are the summary of 2 separate experiments conducted in triplicates; results are represented as mean± SEM. Overall platelet particle production begins at day 8 of the megakaryopoiesis culture. (B) A flow diagram of two time points of the megakaryopoiesis culture, demonstrating the absence and presence of platelets detected on day 0 (post-expansion) and day 8 respectively (highlighted in red).

Detecting antiplatelet autoantibodies in patient plasma and testing its role in the inhibition of megakaryopoiesis.

To compare this reduction in platelet production to antiplatelet autoantibodies levels, all ITP plasma samples were screened in the indirect antigen capture assay. From this assay, six out of twelve samples tested positive for antiplatelet autoantibodies with the following breakdown: two samples tested positive for anti-GPIIb/IIIa only, one for anti-GPIb only and three for both. When comparing normalized platelet production only

samples positive for anti-GPIb (with or without anti-GPIIb/IIIa) had a clear reduction in platelet level (<90%) and this effect was only present in three of those four samples (Figure 4.5). When comparing the platelet to megakaryocyte ratio six of seven plasma samples positive for antiplatelet antibodies (85%) and four out of six (66%) negative plasma samples retained that inhibition.

To determine whether antibodies have a role in affecting platelet production, three different ITP plasma samples were treated with protein G sepharose beads and depleted of the IgG antibody fraction (Figure 4.6). The samples were cultured and compared to sepharose sham controls for reference. One of the three samples demonstrated a large improvement in platelet production when compared to the Sepharose control, increasing platelet levels by 224±23% in comparison to the bead control ($P<0.05$ student T-test). No significant improvement was observed with the other two samples.

To compare autologous vs non-autologous results, three random ITP plasma samples were retested using HSPC from a healthy donor (blood type O) and measured for CD41a+ and CD41aCD42b+ cell growth along with platelet production. As shown in Figure 4.7, differences can be detected between all three measurements with variable effects. These findings demonstrate that testing samples with different HSPC donor cells would not yield the same results as testing samples autologously.

A B

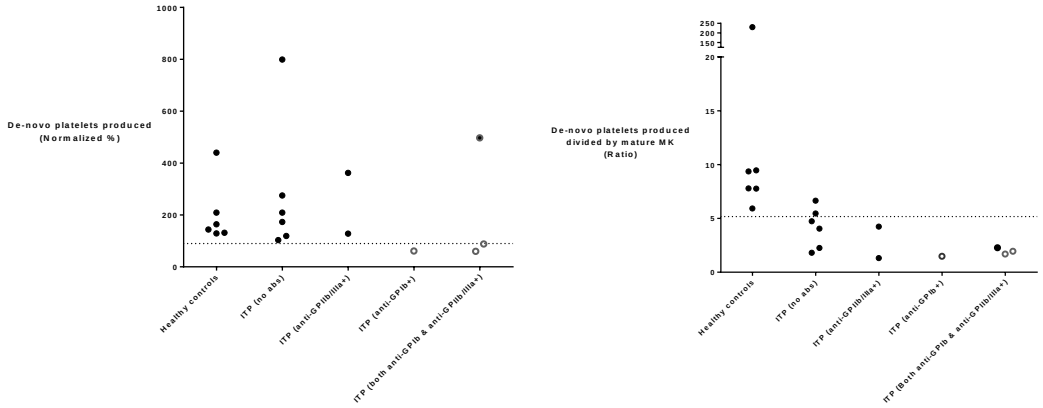

Figure 4.5: Separating plasma impact on platelet production according to antibody presence. The autologously tested plasma was screened for anti-GPIIb/IIIa and anti-GPIb antibodies using the indirect antigen capture assays. Results were then categorized into following groups: Healthy controls, negative for anti-GPIIb/IIIa and anti-GPIb autoantibodies, positive for anti-GPIIb/IIIa autoantibodies, positive for anti-GPIb antibodies, and both meaning the samples were positive for both types of autoantibodies. Results were broken down into either the normalized platelet count in comparison to the medium control (A) or according to the platelet to megakaryocyte ratio (B). Repeated samples are highlighted by open circles. Each dot is the mean of a set of triplicates.

A B

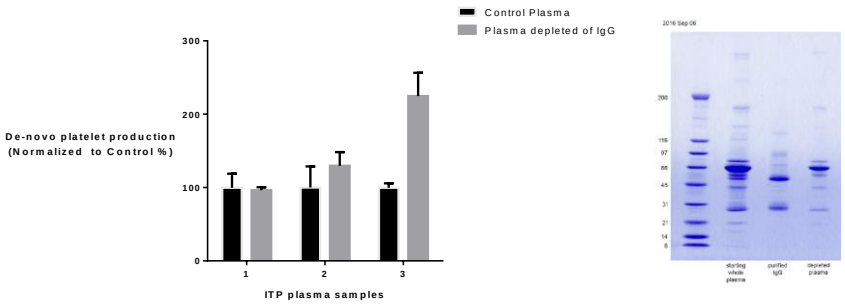

Figure 4.6: ITP plasma depleted of IgG antibodies can improve thrombopoiesis in some ITP samples. To determine whether the inhibition in platelet production is antibody mediated, three inhibiting samples were treated with protein G coated beads and depleted of the antibody fraction. The samples were then tested autologously in the megakaryopoiesis assay and compared to a sepharose control group. As shown above, one sample showed an improvement in platelet production. Antibody depletion was estimated at 90% or higher. A representative SDS-PAGE depicting the depleted plasma and its components is demonstrated in (B).

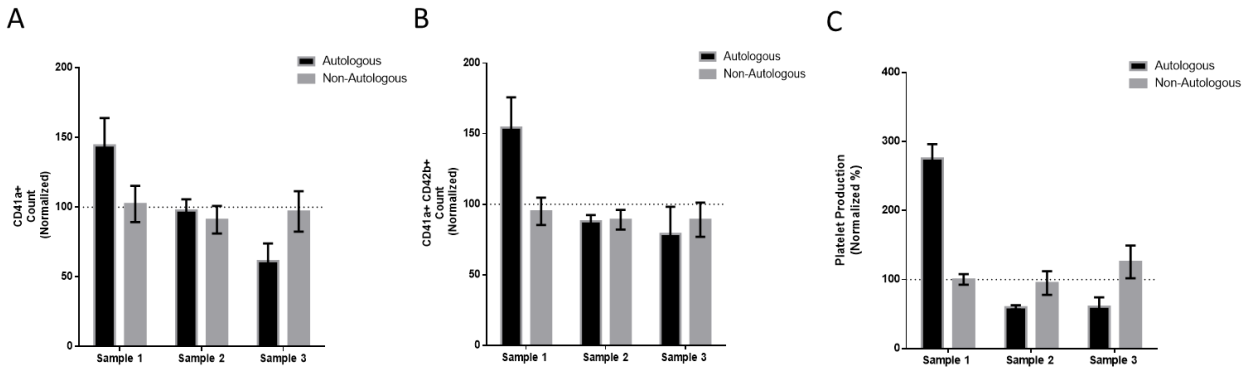

Figure 4.7: Testing ITP plasma autologously vs non-autologously for effect
Three ITP plasma samples that were tested autologously were also tested again with HSPC isolated from a healthy donor (blood group O) in a non-autologous culture to determine whether this factor would affect cell growth. As shown, the effects are varied and differ between samples. Sample 1 shows elevated CD41a Cell growth (A), megakaryocyte maturation (B) and thrombopoiesis (C) potential in the autologous culture, but this was not detected in the non-autologous culture system. Sample 3 shows inhibition in CD41a growth and thrombopoiesis, but this was not detected in the autologous system. Sample 2 was comparable between autologous and non-autologous samples. These results are represented as the mean± standard deviation of three experimental triplicates.

Reproducibility of results

To assess the reproducibility of plasma effects, five plasma samples were collected from a healthy donor, taken at least one month apart, and tested in parallel in the megakaryopoiesis assay. As shown in Figure 4.8, the effects of the plasma were consistent with a variance of 12% for CD41a cell growth, 15% for CD41aCD42b+ cell growth, and 6% for platelet production. To assess the variability of HSPC, a single healthy donor was bled on 5 different occasions and the isolated HSPC was assessed for megakaryopoiesis and thrombopoiesis potential. As shown in Figure 4.8, a high degree of variability was detected between different isolations indicating that the isolated HSPC's

potential for megakaryopoiesis fluctuates considerably between isolations. Overall, variance for HSPC was 50% for CD41a, 63% for CD41aCd42b and 74% for platelet production.

From three separate isolations taken from the same blood donor, the plasma sample taken at the time of blood draw was retested in the autologous megakaryopoiesis assay to determine the variance in plasma for its effect on CD41a and CD41a CD42b cell growth and platelet production. As shown in Figure 4.8, when normalized to the medium control, the coefficients of variation were 20% for CD41a growth, 47% for CD41aCd42b+ and 16% for platelet production. The ratio of platelets to megakaryocytes was found to have a high degree of variability (90%). This result is likely due to being a calculation of two different variables combined.

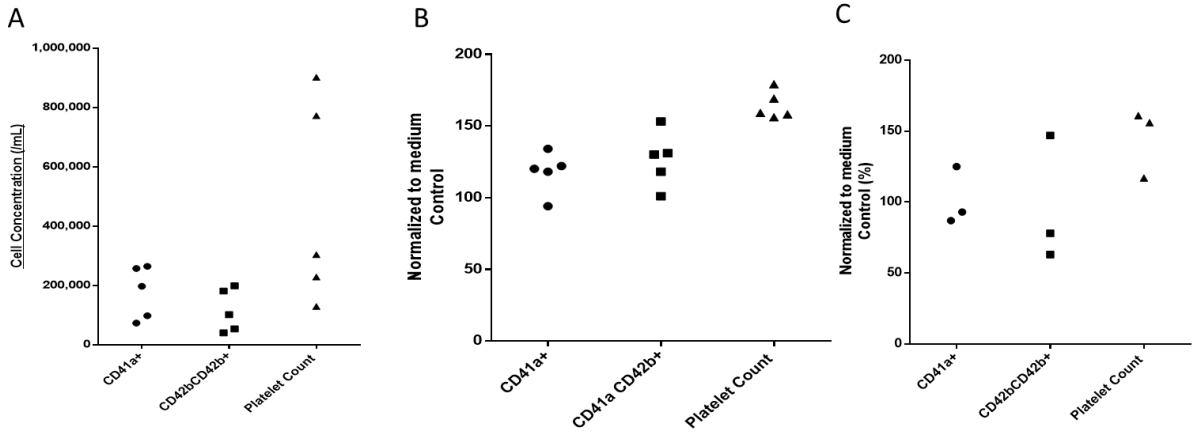

Figure 4.8: Experimental reproducibility of plasma results
(A) Five plasma samples collected at least one month apart from a healthy donor were tested in a single megakaryopoiesis assay to assess the variation of plasma effects on megakaryopoiesis. Results demonstrate that when normalized to the medium control, overall variation caused by the plasma was low with calculated coefficient variations of 12% for CD41a cell growth, 15% for CD41aCD42b+ cell growth, and 6% for platelet production. Each dot represents the mean of an experimental triplicate.

(B) To assess the variation of HSPC potential for megakaryopoiesis, five separate isolations from a healthy donor were performed at least one month apart and their potential for megakaryopoiesis was measured. A high degree of variability was detected between different isolations, indicating the isolated HSPC potential for megakaryopoiesis fluctuates considerably between isolations. Overall variance for HSPC was 50% for CD41a cell growth, 63% for CD41aCd42bcell growth and 74% for platelet production. Each dot represents the mean of an experimental triplicate.
(C) Three separate isolations and autologous plasma samples were tested from a single donor and when normalized to the medium reference, the coefficients of variation were 20% for CD41a growth, 47% CD41aCd42b+ and 16% for platelet production amongst repeat experiments.

Inherent Megakaryocyte growth and thrombopoiesis capacity of ITP HSPC

To analyze the inherent potential of patient HSPC to undergo megakaryopoiesis following TPO stimulation, the percentage and concentration of CD41a+ and CD41a+ CD42b+ megakaryocytes were measured after 8 days of culture at 6 ng/ml of TPO and 50 ng/ml of SCF. In comparison to healthy controls, ITP expanded HSPC do not display any significant reduction in megakaryopoiesis or thrombopoiesis capacity. Overall 67±12% of the cultured HSPC from ITP patients was CD41a+ with the average cell concentration being 180,000 CD41a+ cells/ml. For healthy controls, 57±12% of those cells were CD41a+ and the average culture concentration was 90,000 cells/ml (Figure 4.9). Four of the eleven ITP samples showed an elevated level of CD41a+ cell growth (>200,000 CD41a+ cells/ml), with the highest sample originating from a patient being treated with a TPO mimetic. There was no statistical significance detected between ITP and healthy donors' experiments as a group.

Similar trends were found when measuring the mature megakaryocyte population (CD41a+, CD42b+) as a subgroup of the ITP HSPC displayed an elevated potential to grow mature megakaryocytes (Figure 4.10). Overall 54±15% of the cultured

megakaryocytes (CD41a+) were positive for CD42+ with an average cell concentration of 110,000 cells/ml in culture. In comparison to healthy controls, 46±16% of culture megakaryocytes were CD42b+ with an average cell count of 50,000 mature megakaryocytes per ml. When applying a two standard deviation cut-off from the healthy controls (>100,000), it was observed that the same four samples that had elevated $CD41a^{+}$ cell counts also had elevated mature megakaryocyte cell levels (Figure 4.10). The difference in mature megakaryocytes between ITP and HC group was statistically significant (Mann-Whitney U test p=0.03).

Platelet production was measured with the results summarized in Figure 4.11. One of the healthy controls had an elevated platelet count that was found to be statistically significant from the remaining cell groups (platelet count > $2*10^6$ platelet/ml, Grubbs test $P<0.05$). When removed from the analysis and a two standard deviation cut-off from the remaining healthy controls was applied, it was determined that two of the ITP samples had an elevated platelet count. Both samples were from experiments showing elevated megakaryocyte maturity and cell count number. No correlation between the number of cultured megakaryocytes, their maturation status, or the number of platelets produced was connected to the thrombocytopenic status of the patient at the time of blood collection. Elevated megakaryocyte numbers and maturation did not correspond to TPO mimetic treatment.

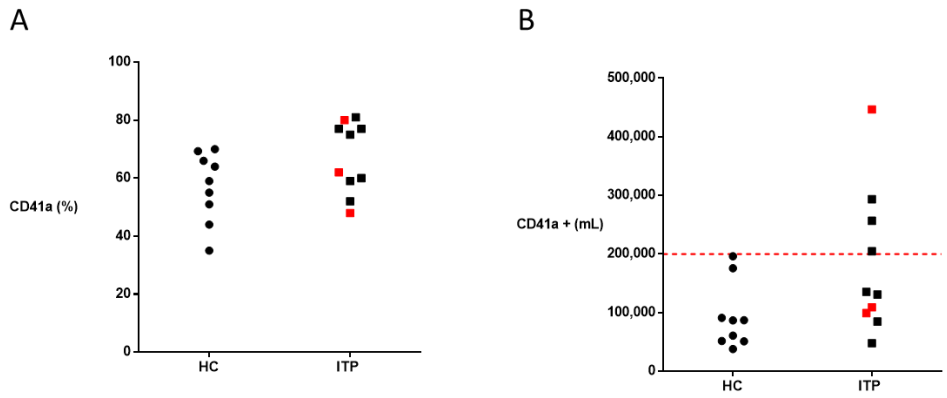

Figure 4.9: Megakaryocyte growth is comparable between ITP and healthy donor HSPC Isolated HSPC from ITP and healthy samples (HC) were stimulated with 6 ng/ml of TPO and 50 ng/ml of SCF and cultured for 8 days. (A) The expression of CD41a+ was comparable between the two groups. (67±12% vs 57±12%) (B) The CD41a+ cell count was elevated in four of the ten ITP samples as indicated by the two standard deviation cut-offs marked from the healthy donor group (highlighted by the dotted line). Each dot represents the mean of a set of triplicates. Samples highlighted in red were from patients undergoing TPO mimetic treatment at the time of isolation

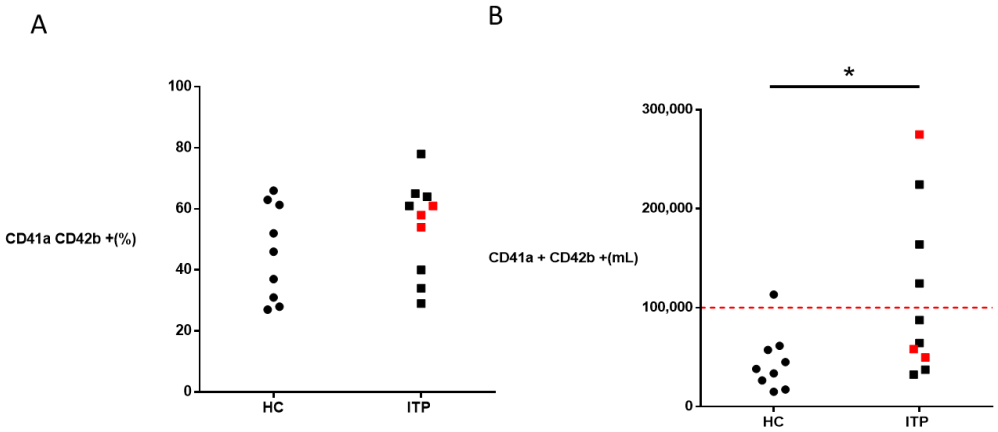

Figure 4.10: Mature megakaryocyte growth was increased in a subgroup of ITP HSPC Isolated HSPC from ITP and healthy samples (HC) were stimulated with 6 ng/ml of TPO and 50 ng/ml of SCF and cultured for 8 days. (A) Expression of CD41a+ CD42b+ was comparable between ITP and HC groups (54±15% vs 53±15%). (B) The absolute CD41a+ CD42b+ cell count was increased in four out of ten ITP samples. A two standard deviations cut-off above the healthy donor group is highlighted by the dotted line. Each dot represents the mean of a set of triplicates.

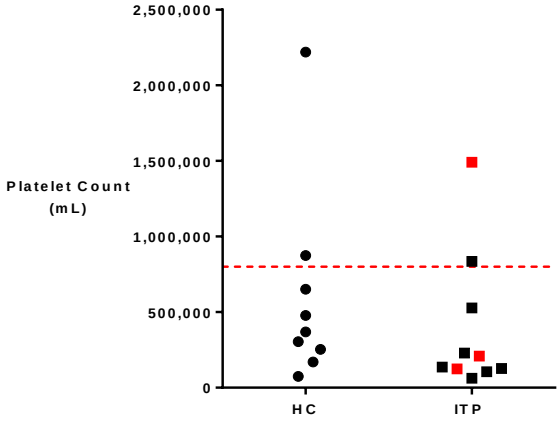

Figure 4.11: Platelet production was increased in only two of the ITP patient samples
Isolated HSPC from ITP and healthy samples (HC) were stimulated with 6 ng/ml of TPO and 50 ng/ml of SCF and cultured for 8 days before the cultured platelet count was measured. Two of the eleven ITP cultures demonstrated an increase in platelet production in comparison to healthy controls. One healthy control was determined to be an outlier as determined using the Grubs test and was excluded from establishing the two standard deviations cut-off from the healthy controls (dotted line). Each dot is the mean of a set of triplicates. Samples highlighted in red were from patients undergoing TPO mimetic treatment at the time of isolation

Testing Glanzmann plasma for inhibition of platelet production

In addition to the ITP plasma samples, a Glanzmann plasma sample that was positive for anti-GPIIb/IIIa polyclonal antibodies was also tested in the megakaryopoiesis assay. The addition of the plasma sample resulted in the mature megakaryocyte cell population (CD42b+) reduced to a fraction of a pooled normal plasma control (25% from an average of 2 separate experiments done in triplicates). It was determined that this effect was also present when the plasma was added to a mature megakaryocyte population causing severe agglutination within 40 minutes of incubation (Figure 4.12). The number of cellular clumps increased by 200% in comparison to the autologous control plasma and a reduction in the CD42b cell count to 41±4% of the reference control

(Figure 4.12). The addition of the plasma caused no changes in p-selectin expression indicating no activation was present (Figure 4.12).

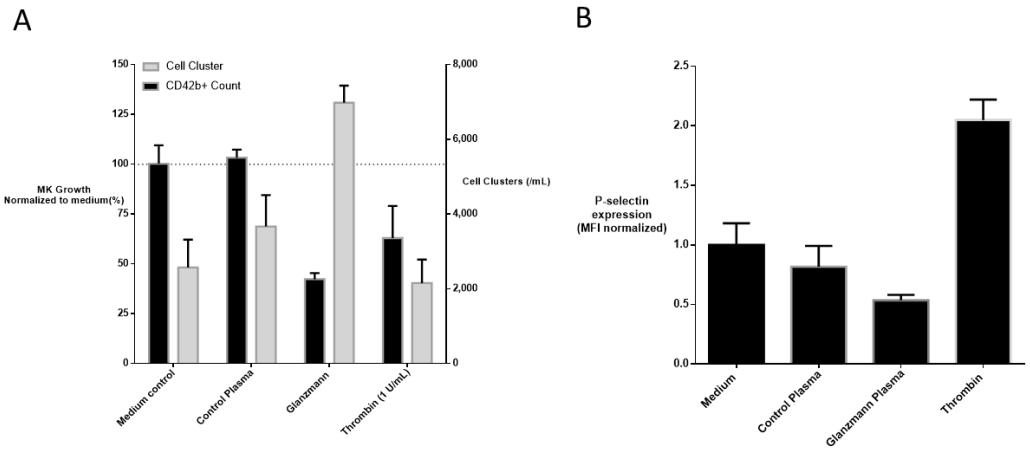

Figure 4.12: Glanzmann Plasma agglutinates mature megakaryocytes without cellular activation Glanzmann plasma was added to mature megakaryocytes at 10% by volume and incubated for 40 minutes at 37ºC before analysis on the flow cytometer to determine its immediate impact on the number of mature megakaryocytes (A) and changes in p-selectin expression as measure by CD62P MFI (B). A thrombin concentration of 1 U/ml was used as a positive control. No analysis using CD41a was possible as the polyclonal anti-GPIIb/IIIa plasma blocked all detecting flow cytometry antibodies. These results are representative of a single experiment conducted in experimental triplicates and plotted as the mean± standard deviation. All experiments were repeated twice using blood matched healthy donor HSPC with the control plasma representing an autologous sample collected from the same donor.

4.6 Discussion

Immune thrombocytopenia is a heterogeneous autoimmune blood disorder involving the immune system destroying platelets and inhibiting platelet production.[29,30] Despite evidence demonstrating inhibition of platelet production, the mechanism is poorly understood, and the clinical usefulness of such information has not been

established. To our knowledge, this report represents the first study testing ITP patient plasma to investigate its effect on megakaryopoiesis and thrombopoiesis in an autologous cell culture assay using the patient's HSPC. We also assessed the inherent potential of ITP patient HSPC to undergo megakaryopoiesis & thrombopoiesis. The goal was to identify and characterize any inhibition or defects in platelet production caused by patient plasma, as well as validate the potential use of this peripheral blood-based megakaryopoiesis assay to study platelet production in ITP.

The addition of ITP plasma did not inhibit megakaryopoiesis in our autologous assay. In some samples, we found that the addition of plasma increased the number of megakaryocytes generated. This effect was especially noticeable for the mature megakaryocyte population in that 30% of plasma samples tested caused a significant increase in that cell population. Similar effects have been observed in other ITP studies where it has been shown that some ITP plasma samples support the development of megakaryocytes.[19,13] Our results contrast with previous reports demonstrating inhibition of megakaryopoiesis and this can be attributed to two different factors: either the small sample size of patients tested or the lack of additional immune components such as the complement system or the need for additional immune cells necessary to cause an effect.[13,14,31] Some of the inhibition detected in previous investigations reported that the addition of a plasma component, likely the complement system, was necessary for inhibition.[14] As our samples were heat-treated this factor would have been removed in this study. Our allogeneic vs. autologous cultures also show considerable variability

between experiments and should serve to caution interpretations of inhibition in non-autologous cell assays where other factors such as alloimmune antibodies could have an impact.[32]

The addition of ITP plasma did have a significant inhibiting effect on thrombopoiesis. We found that six of twelve plasma samples had platelet levels lower than the lowest healthy control; three of which were at least two standard deviations below the control group. Relative to the number of megakaryocytes cultured, there was a lower than expected number of platelets present in most ITP experiments (8/11) in comparison to healthy donor cultures. This inhibition was partially antibody mediated, as antibody absorptions reversed this effect in a test sample. This result aligns with previous studies showing inhibited proplatelet formation following the addition of ITP patient plasma or autoantibodies to mature megakaryocytes.[19,11,10] This effect on thrombopoiesis and not megakaryopoiesis suggests that ITP plasma is inhibiting the megakaryocyte's ability to release platelets. Certain plasma proteins such as fibrinogen, type I & IV collagens and interferons are known to either suppress or support proplatelet formation in megakaryocytes. [33–36] It may be possible that ITP autoantibodies are blocking or suppressing the receptors responsible for thrombopoiesis or altering their glycosylation status affecting their function as has been recently reported.[37] This action might halt the megakaryocyte from producing platelets but allow it to remain in the bone marrow. This could explain why impaired platelet production is present in ITP patients while no change in megakaryocyte levels are detected in bone marrow studies or in-vivo mouse models.[38–

[40] This could also explain why there are no elevated TPO concentrations in most ITP patients as the trapped megakaryocytes would continue consuming TPO and so create an imbalance in the platelet production cycle.[41] This platelet production inhibition was present with samples that tested positive for anti-GPIb autoantibodies but not every plasma with this autoantibody had this effect. This could be the result of concentration differences or antibody specificity to different epitopes being targeted by these autoantibodies. Some of these autoantibodies could also be irrelevant by-products generated by excessive platelet destruction as has been suggested by previous ITP studies.[42]

The inherent potential of patient HSPC to undergo megakaryopoiesis was measured and it was observed that a subset had an enhanced capacity to generate megakaryocytes. Four of the ten HSPC samples isolated from ITP patients generated significantly more megakaryocytes (both CD41a+ and CD41a+CD42b+ progenitors) than what was observed with the healthy donor controls despite identical TPO concentrations and culture conditions. This observation was present without the addition of patient plasma, suggesting the HSPC were already been primed for megakaryopoiesis. This finding did not correspond with samples collected from patients taking TPO mimetics nor with the initial platelet count at the time of blood draw. This enhanced megakaryopoiesis subgroup could be representing the ITP patients reported having increased platelet production in an effort to maintain hemostasis or those with elevated TPO levels in circulation.[36,9] It has been shown that certain early stem cell progenitors can skip certain

developmental stages or checkpoints and go directly into megakaryopoiesis and perhaps this could be what occurred with these patients' cells.[43,44] Recent investigations regarding platelet glycosylation also suggest that impaired platelet production in ITP could be a result of a lack of TPO production and so stimulation of megakaryopoiesis is what is missing to increase platelet production.[45,46] In this case, this enhanced potential for megakaryopoiesis from some patient HSPC could be a natural response to a thrombocytopenic environment and the "normal" amount of megakaryocytes generated in other ITP cultures could from patients who have failed to elevate platelet production in an effort to maintain hemostasis.

In addition to the ITP samples tested, we reported the results of a polyclonal anti-GPIIb/IIIa Glanzmann plasma sample and its inhibitory effect on megakaryopoiesis. We determined the effect was immediate and suspect agglutination was present as large cell clustering was detected without any indication of platelet activation. If true this effect is unlikely to be present in vivo as the frequency of megakaryocytes in the bone marrow is low (~0.03%) making such interacts unlikely to occur.[47] This result shows that anti-GPIIb/IIIa antibodies can cause severe agglutination in vitro and that such antibodies do exist in patient plasma. Future work investigating anti-GPIIb/IIIa antibodies for the inhibition of megakaryopoiesis should consider this.

The main limitation of this study is its small sample size and the lack of non-immune thrombocytopenic controls. The addition of non-immune thrombocytopenic

samples would determine whether the enhanced megakaryopoiesis potential found in some ITP HSPC is a natural response during a state of thrombocytopenia or whether this enhanced effect is exclusively present in ITP pathology. In addition, there was a higher failure rate of HSPC isolations in ITP patients than in healthy controls, which was not associated with platelet count or medication use. Further refinement of the isolation protocols may be necessary to achieve higher rates of success. In addition to this, not all antiplatelet autoantibodies were screened and so the role of anti-CMpl or anti-TPO autoantibodies in causing inhibition of megakaryopoiesis is unresolved. [48] The last limitation is the general difficulty in identifying and diagnosing ITP patients in addition to finding samples positive for antiplatelet autoantibodies for testing; the role of the McMaster ITP registry and its large sample repository has been a great help in finding and testing these patients samples.[49,50] Despite these issues the use of peripheral blood as a source to grow autologous megakaryocytes from ITP patients is possible as we have demonstrated HSPC can be isolated from ITP patients and grown into mature megakaryocytes.

In summary, we demonstrate no inhibition of megakaryopoiesis with ITP plasma using autologous cultured megakaryocytes; however, a reduction in thrombopoiesis was observed. We found that culturing megakaryocytes from peripheral blood CD34+ cells isolated directly from ITP patients was feasible. Such megakaryocytes can be used to study complement and immune cell interactions with megakaryocytes in a completely

autologous setting and can allow for further exploration into platelet production in ITP patients.

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4.8 Contribution:

NI carried out the described studies, analyzed data, and wrote the manuscript. IN designed the research, interpreted data, and wrote the manuscript. RC and DMA, MS provided clinical information. JWS performed SDS. NI designed the research and wrote the manuscript. All authors reviewed and approved the final version of the manuscript

4.9 Disclosure of conflicts of interest

The other authors state that they have no conflict of interests.

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Table 4.1: Clinical summary of ITP patients including their medication and platelet count at the time of blood collection.

ITP	Diagnosis	platelet count (10^9/L)	Medication of Patient	Age	Gender	Anti-GPIIb/IIIa	Anti-GPIb
1	Primary ITP	46	IVIG	28	M	-	-
2	Primary ITP	252	Nplate, prednisone	74	F	-	-
3	Primary ITP	145	none	32	F	-	-
4	Primary ITP	15	prednisone, omeprazole, cellcept, azathioprine	87	M	-	-
5	Primary ITP	6	prednisone	63	M	+	-
6	Primary ITP	5	none	56	M	-	-
7	Primary ITP	77	none	40	M	-	-
8	Primary ITP	106	Eltrombopaq, Azathioprine	52	M	n.t.	n.t.
	Repeat ITP	101	Azathioprine			+	+
	Repeat ITP	91	none			+	+
9	Primary ITP	34	Eltrombopaq, risedronate, atorvastin	68	F	+	-
10	Primary ITP	75	Mycophenolate mofetil	65	M	+	+
	Repeat	61	none			-	+

n.t. sample not tested; repeated samples are highlighted to the corresponding patient

Table 4.2: A summary of the isolation and expansion of HSPC from ITP patient peripheral blood

	ITP patients	Health controls
Number of Donors	10	9
Blood Volume (ml)	61±10	79±8
HSPC Yield (per ml of blood)	2200±1100	1500±900
Initial Purity (%)	84±6%	79±9%
Initial Cell Viability (%)	74±14%	61±17%
Fold Expansion of HSPC	5±1	7±2
Expanded HSPC Purity (%)	92±4%	92±4%

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Chapter 5

5.1 Conclusion

This thesis investigates the mechanisms through which ITP patient plasma and antiplatelet antibodies inhibit platelet production by megakaryocytes. ITP is a heterogeneous immune-mediated blood disorder with multiple pathogenic mechanisms causing thrombocytopenia.[73] There are no reliable biomarkers to identify possible ITP cases and the ability to predict the development of symptoms or to predict patient responses to treatments are currently major hurdles in the diagnosis and treatment of ITP. I established an autologous peripheral blood-based megakaryopoiesis assay for studying the mechanisms through which ITP plasma inhibits platelet production. Using this assay, I determined that most ITP patient plasmas impair thrombopoiesis, but did not affect megakaryopoiesis. I found that a subset of ITP patients had HSPC with enhanced potential for megakaryopoiesis, which has important implications for measuring the platelet production capacities of ITP patients. I also identified a novel monoclonal anti-

GPIb antibody that impairs megakaryocyte maturation, which can be used as a model for further inhibitory studies to study the pathogenesis of ITP. The results described in this thesis enhance our understanding of the etiologies of ITP in a subset of patients with antiplatelet autoantibodies, which contributes to a better classification of ITP patients and more effective treatment plans.[53,58,62]

The principal cause of thrombocytopenia in ITP is thought to be platelet destruction by antiplatelet autoantibodies.[71,72,74] Megakaryocytes, the bone marrow cells that produce platelets, express the same platelet receptors recognized by ITP autoantibodies, and it has been hypothesized that antiplatelet autoantibodies also target and inhibit megakaryocytes and impair platelet production. [36,72,117] Platelet turnover studies show a reduction in platelet production in some ITP patients, and *in vitro* cell, culture assays show that the addition of ITP plasma and antiplatelet autoantibodies can inhibit megakaryopoiesis and/or thrombopoiesis. [72,96,98,99,101,104,105,118,119] However, bone marrow studies and animal models show no change in megakaryocyte levels, which contrast with *in-vitro* cell culture results.[113,115]

Presently, the identity and mechanism of how antiplatelet autoantibodies and ITP plasma inhibit platelet production have not been conclusively determined. Previous megakaryopoiesis assays used to study the effects of antiplatelet antibodies on platelet production were also allogenic, testing plasma samples with cells from a different donor. As a result, other factors such as HLA and HPA antibodies that are not relevant in ITP

pathology could cause misleading and undesirable side effects and make it difficult to draw a conclusion. I established an autologous megakaryopoiesis assay that circumvents inter-species and inter-individual interference in determinations of the effect of these autoantibodies on platelet production by cultured megakaryocytes. A major challenge in studies of ITP megakaryocytes is sample acquisition. The method developed herein uses peripheral blood as a source of HSPC for megakaryopoiesis instead of bone marrow or cord blood sources. Furthermore, these HSPC are expanded in culture to reduce the amount of blood required from ITP patients for experiments. In this model, these expanded HSPC are TPO-receptive and can develop into mature megakaryocytes that can undergo thrombopoiesis. This autologous megakaryopoiesis assay can be used to study any form of immune effect caused by ITP plasma, autoantibodies, and autoreactive immune cells from the patient population as well as investigate the inherent capacity of patient HSPC to develop into megakaryocytes.[120]

The development of this autologous assay has several important implications that extend beyond the scope of this project and open new avenues of investigation regarding the various interactions megakaryocytes have in the bone marrow. Megakaryocytes communicate with numerous different cells during their development, being both affected by and influencing the development of neighbouring cells as they migrate across various niches in the bone marrow.[121] These interactions not only include bone marrow cells like osteoblasts, osteoclasts, mesenchymal stem cells and HSPC but also immune cells like B cells and macrophages; where megakaryocytes can influence their activation status and

development.[121–125] Since this assay is autologous, the possibility of studying these natural interactions would allow a more complete understanding of megakaryocytes and the effect they have on these residing cells. Studies could also be conducted to investigate how different compounds (ranging from drugs like immunosuppressants to various extracellular matrix proteins) would affect these interactions, and what is their downstream significance on this form of cellular communication.[125–127] Since this assay uses peripheral blood, it can be expanded to study patients with other deficiencies or defects in megakaryocytes or other bone marrow cells, including everything from different cancers to various genetic mutations or polymorphisms to determine what effect these factors would have on megakaryocytes and their role in the bone marrow.[128–130] The potential applications this developed assay has are numerous and pave the way for further investigations regarding the influence and effects megakaryocytes have in the bone marrow environment.

Using this developed assay system, I investigated the effect ITP patient plasma had on platelet production using each patient's own HSPC. There was no inhibition of megakaryopoiesis following the addition of the patient's plasma. The number of megakaryocytes cultured was comparable to healthy controls or, in some cases, higher. This trend was more prevalent when analyzing the mature megakaryocyte population and did not correspond to samples from patients who were taking TPO mimetics. The only inhibition observed following the addition of patient plasma was a reduction in the number of platelets detected. There was a disproportionately lower number of platelets

relative to megakaryocytes present in most ITP cultures in comparison to healthy controls. These data demonstrate that ITP plasma affects the ability of cultured megakaryocytes to produce platelets and that this could be more important than the number of megakaryocytes cultured. This effect is in part antibody-mediated and suggests that perhaps the autoantibodies are preventing megakaryocytes from undergoing thrombopoiesis. [26] This result implies that the inhibiting effect caused by ITP plasma is more of a functional effect rather than a direct reduction of megakaryocyte growth and that future work should focus on refining its effects on thrombopoiesis. These results match recent observations showing no changes in megakaryocyte bone marrow samples but explain why there is a reduction in platelet production.[119,131,132] At present, a major limitation of this study is the low sample size and the lack of additional immune components (i.e. complement, other immune cells, etc.) that could affect platelet production.[133,134] In future experiments, testing cultured megakaryocytes from a larger sample of ITP patients will likely reveal differential effects of autologous plasma on cultured megakaryocytes, reflecting the multiplicity of causes of thrombocytopenia in ITP. Adding back other immune components –complement factors, T cells, macrophages – may reveal additional mechanisms through which the immune system affects platelet production by megakaryocytes. [124,133–135]

Another key observation from these experiments was that some ITP HSPC generated more megakaryocytes compared to healthy donor HSPC. This effect was present without the addition of plasma and at identical TPO concentrations, which

suggests that some ITP patients have HSPC that were already "primed" for megakaryopoiesis. These particular patients could be representing the subgroup of ITP patients shown to have increased platelet production in an effort to maintain hemostasis.[96] By identifying this ITP subgroup and screening to determine if there is a difference in the type of HSPC progenitors isolated, we may be able to separate patients based on their HSPC capacity for megakaryopoiesis and determine whether this would be a useful biomarker for identifying different platelet production levels.

In addition to testing ITP plasma, I tested monoclonal anti-GPIIb/IIIa and anti-GPIb antibodies for inhibition of megakaryopoiesis. From the panel of anti-GPIIb/IIIa antibodies tested, the monoclonal antibody Raj-1 was the only one that had an effect, and this was due to cell agglutination. Importantly, this agglutination can be misinterpreted for inhibition of platelet production if cultured over an extended time, and this process is unlikely to occur in patients *in vivo*. Agglutination was also present when testing Glanzmann's thrombasthenia plasma, demonstrating that patients can also develop such antibodies *in vivo*. When testing anti-GPIb monoclonal antibodies, I identified an antibody (TW-1) that inhibited megakaryocyte maturation. This inhibition was also present when testing the Fab antibody fragment, suggesting intracellular signalling events are involved in causing this effect. Future experiments to determine the mechanism through which TW-1 causes inhibition of megakaryocyte maturation could be useful for determining the cause of ITP in patients whose thrombocytopenia is characterized by impaired megakaryocyte maturation. Observations of TW-1 interactions with cultured

megakaryocytes also provide direct evidence that antiplatelet antibodies can affect megakaryopoiesis.

In summary, I created an autologous megakaryopoiesis assay and used it to investigate the effect ITP plasma has on platelet production. I found inhibition of platelet release to be the main negative effect caused by ITP plasma. In addition, a model inhibiting monoclonal antibody was identified and evidence suggesting a difference in patient HSPC to develop into megakaryocytes was uncovered. This thesis has developed the tools necessary for autologous investigations of impaired platelet production in ITP and provides direct evidence that impaired platelet release is the main effect caused by ITP plasma.

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