A MEGAKARYOCYTE CULTURE FROM PERIPHERAL BLOOD HEMATOPOIETIC PROGENITOR CELLS FOR THE INVESTIGATION OF PLATELET DISORDERS

THE DEVELOPMENT AND OPTIMIZATION OF A HUMAN MEGAKARYOCYTE CULTURE FROM HEMATOPOIETIC PROGENITOR CELLS ISOLATED FROM NORMAL PERIPHERAL BLOOD FOR *IN VITRO* INVESTIGATION OF PLATELET DISORDERS

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Abstract

Megakaryocyte cultures are a strong tool for the *in vitro* investigation of platelet production in platelet disorders. Megakaryocytes are platelet precursors which are produced in response of thrombopoietin (TPO) in the bone marrow from hematopoietic progenitor cells in a process called megakaryopoiesis. Peripheral blood derived hematopoietic progenitor cells (PB-HPCs) are the most accessible source of HPCs with high potential to produce mature and functional megakaryocytes *in vitro*; however, they are present in low numbers making peripheral blood an inefficient source for large scale experiments Additionally, a megakaryocyte culture with an optimized TPO concentration is required which can reliably allow the investigation of suppressive effects of antibodies/plasma from immune thrombocytopenia (ITP) patients. In this study, we developed a megakaryocyte culture with the utilization of human PB-HPCs in an efficient fashion resulting in the production of high purity megakaryocytes in a TPO-dependent manner.

The mononuclear fraction was collected from 180 mL of peripheral whole blood and CD34+ cells were isolated by a positive selection yielding the average of 5.5 x 10^5 ± 2.5 x 10^5 CD34+ cells (n = 18). Using 96-well tissue-culture plates and seeding 10,000 CD34+ cells/well, the average of 13 experiments in triplicate can be set up utilizing isolated CD34+ in an efficient manner. No correlation was found between the purity of CD34+ cell samples and megakaryocyte production. Therefore no cutoff for CD34+ cells purity could be established. Capitalizing on a

TPO dose-dependent megakaryocyte production experiment, 20 ng/mL was established as the TPO concentration which resulted in the production of mature megakaryocytes without reaching the plateau in megakaryopoiesis response. On day 11 of culture, the expression of megakaryocytic lineage (CD41/61+) and maturation (CD41/61+CD42+) markers peaked at 90.65% and 76.10%, respectively with 4.2% of megakaryocytes reaching16N polyploidy levels. In conclusion, this culture system has broad application for investigation of platelet disorders and drug discovery which can be accessible to all researchers.

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ABBREVIATIONS

ACD (Acid citrate dextrose)

BM-HPCs (Bone marrow)

BSA (Bovine serum albumin)

CD (Cluster of differentiation)

CB-HPCs (Cord blood hematopoietic progenitor cells)

DAPI (4',6-diamidino-2-phenylindole)

DMS (Demarcation membrane systems)

DNA (Deoxyribonucleic acid)

EDTA (Ethylenediaminetetraacetic acid)

FITC (Fluorescein isothiocyanate)

gp (Glycoprotein)

G-CSF (Granulocyte-colony stimulating factor)

GM-CSF (Granulocyte-monocyte colony stimulating factor)

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

Hematoxylin and eosin (H and E)

HPCs (hematopoietic progenitor cells)

IgG (Immunoglobulin G)

ITP (Immune thrombocytopenia)

IL-3 (Interleukin-3)

IL-6 (Interleukin-6)

IMDM (Iscove's Modified Dulbecco's Medium)

Minutes (min)

MNCs (Mononuclear cells)

PB-CD34+ cells (Peripheral blood CD34+ cells)

PBMCs (Peripheral blood mononuclear cells)

PB-HPCs (Peripheral blood hematopoietic progenitor cells)

PBS (Phosphate buffer solution)

PFA (Paraformaldehyde)

PE (Phycoerythrin)

PE-Cy5 (r- Phycoerythrin cyanine 5)

PRP (Platelet rich plasma)

PI (Propidium iodide)

PGE1 (Prostaglandin E1)

RNA (Ribonucleic acid)

SCF (Stem cell factor)

TPO (Thrombopoietin)

vWF (von Willebrand factor)

TRITC (tetramethylrhodamine-5-(and-6)-isothiocyanate)

CHAPTER 1: INTRODUCTION

1.1 Platelet Production

1.1.1 Platelets:

Platelets have an essential role in primary hemostasis and are very important in the prevention of bleeding. Upon vascular injury, platelets change their shape, become activated, bind to the site of injury and provide a charged surface to initiate and support coagulation events (Rodak et al., 2007). Platelets are anucleated with average diameter size of 2.5 µm and the platelet normal range of 150-400 x 10^9 /L in peripheral blood in healthy individuals (Rodak et al., 2007). Decreased number of peripheral platelets below the normal range is associated with an increased risk of bleeding (Provan et al., 2010). Platelets are produced from a unique type of cells called megakaryocytes in the bone marrow in response to thrombopoietin (TPO) (Rodak et al., 2007). Every day 1 x 10^{11} platelets are produced and an equal number are removed from circulation (Rodak et al., 2007).

1.1.2 The role of thrombopoietin (TPO) in platelet production:

TPO was isolated and purified by several research groups in the early 1990s (de Sauvage et al., Kaushansky et al., Kuter et al., 1994). TPO is a glycoprotein of approximately 35kDa in size consisting of 353 amino acids. The liver and kidneys are involved in TPO production; however, the major site for production of TPO is the liver (de Sauvage et al., 1994). TPO is a growth factor that belongs to the hematopoietic cytokine family and binds to its receptor, c-Mpl

(CD110, the product of human myeloproliferative leukemia virus gene), on the surface of megakaryocytes and hematopoietic stem cells (Kaushansky et al., Zeigler, et al., 1994). Once TPO is bound to c-Mpl, it activates downstream signalling pathways by homodimerization of the c-Mpl receptors (Kaushansky, 2002, Arnold et al., 2009). The c-Mpl homodimer induces the activation of intracellular tyrosine kinase JAK-2, phosphorylation of signal transducers and activators of transcription factor 3 and 5 (STAT3 and STAT5) and subsequently, activation of mitogen activated protein kinase (MAPK) and Phosphatidylinositide 3-kinases (PI3K) (Kaushansky, 2002). The stimulation of these downstream signals results in 1) differentiation and proliferation of megakaryocytes and 2) prevention of programmed cell death in megakaryocytes and HPCs (Kaushansky, 2002). In a study involving c-Mpl/TPO knockout mice, platelets were decreased to 10% of the original platelet number (Bunting et al., 1997); therefore, TPO is considered to be the main regulator of platelet production; however a small number of platelets can still be produced in the absence of TPO (Arnold et al., 2009, Kuter et al., 1994). Platelets are the regulators of the TPO levels: excess TPO in plasma binds to c-Mpl on circulating platelets (there are approximately 15-35 TPO receptors on each platelet) and the c-Mpl/TPO complex becomes internalized and rapidly degraded (Fielder et al., 1996, Kuter et al., 1994, Saur et al., 2010, Kuwaki et al., 1998).

1.1.3 Megakaryocytes and Megakaryopoiesis:

Megakaryocytes have unique features: they are large (30-50 µm in diameter), polyploid and have a low frequency in the bone marrow (less than 0.5-1% of total nucleated cells) (Majka et al., 2001, Rodak et al., 2007). Megakaryocytes are produced from hematopoietic progenitor cells (HPCs) through a process called megakaryopoiesis consisting of two major phases: 1) differentiation and proliferation of megakaryocytes 2) maturation (Majka et al., 2001, Rodak et al., 2007). Megakaryopoiesis starts once regulatory signals from TPO and other growth factors stimulate HPCs to commit to the megakaryocytic lineage by expression of surface glycoprotein (gp) IIb/IIIa complex (CD41/61) (Hoffman, 1996). This glycoprotein is an integrin molecule which functions as the fibrin receptor on platelets and plays a major role in platelet activation during the primary hemostasis response (Rodak et al., 2007). As progenitor cells further differentiate to immature megakaryocytes, the expression level of CD41/61 increases while the markers of HPCs (such as the expression of CD34) decrease (Lepage et al., 2000). As megakaryocytes progress toward maturation, their ability to divide is lost due to endomitosis. During this process, the DNA synthesis continues in absence of cell division which results in an increase in DNA content (Zimmet et al., 2000). Polyploidy, the hallmark of megakaryocyte maturation, is the state of having more than one set of chromosome (>2N) which reaches up to 64N or 128N in fully matured megakaryocytes (Geddis, 2010). It has been shown that higher DNA content is associated with the maturation features in

megakaryocytes (Mattia et al., 2002). Another important maturation characteristic in megakaryocytes is the expression of surface gp Ib (CD42) (Lepage, 2000). This molecule is part of gp Ib-V-IX complex which acts as the von Willebrand Factor (vWF) receptor on platelets and is involved in adhesion of platelets to the site of vessel injury during primary hemostasis (Rodak et al., 2007). Demarcation membrane systems (DMS) are also a unique feature in megakaryocytes and a characteristic of cytoplasmic maturation (Geddis, 2010). DMS is a membrane network extending in to the cytoplasm and supports the formation of proplatelets (Schulze et al., 2006, Geddis, 2010). Proplatelets are long pseudopod-like cytoplasmic branches which are formed from fusion of microtubules -- mainly β-tubulin (Italiano et al., 2003). Proplatelets extend into the sinusoidal blood vessels of the bone marrow and it has been shown that platelets are produced and released from the tip of the proplatelets (Italiano et al., 2003). Megakaryopoiesis stages are summarized and illustrated in figure 1.

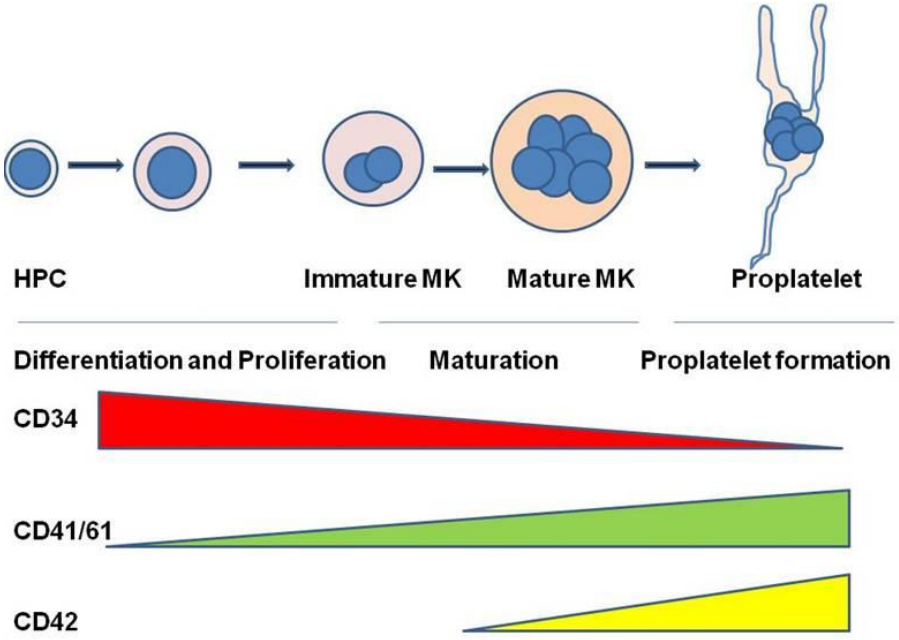


Figure 1: The stages of megakaryopoiesis: megakaryocytes (MK) are produced in bone marrow in response to thrombopoietin (TPO) from CD34+ hematopoietic progenitor cells (HPCs). During differentiation and proliferation, HPCs express the lineage marker, CD41/61 and go through cell division; however, once endomitosis starts, the size and the polyploidy levels in megakaryocytes increase without any further cell divisions. As cells further progress toward maturation, the expression of CD34 decreases while CD41/61 and CD42 expressions increase. A fully matured megakaryocyte produces long pseudopod-like cytoplasmic structures called proplatelets which produces platelets from its tips.

1.2 Megakaryocyte cultures:

Megakaryocyte cultures have been under development and improvement for the past two decades. Megakaryocyte cultures can be used in variety of applications, but the main objectives for developing megakaryocyte cultures

were: 1) exploring platelet production biology 2) *in vitro* investigation of platelet production disorders 3) large production of megakaryocytes for engraftment in patients with platelet production/bone marrow disorders.

In vitro growth of human megakaryocytes has been associated with numerous technical difficulties: Megakaryocytes have low frequency in bone marrow (0.5 - 1% of all nucleated cells, Majka et al., 2001) and are difficult to harvest. Several factors (such as transforming growth factor β (TGF β) present in fetal bovine serum) are inhibitory to the *in vitro* growth of megakaryocytes, and more importantly the primary growth factor which yields pure megakaryocytes *in vitro* was not yet identified (Kaushansky 1999, Majka et al., 2001). Hence, megakaryopoiesis remained a mysterious process and the studies of megakaryocytes were limited to cell lines such as MEG-01 (Kaushansky 1999, Majka et al., 2001). The majority of these cell lines which have been established from patients with leukemia, express the lineage marker, CD41/61; however, the expression of many maturation characteristics such as CD42, VWF and polyploidy cannot be achieved by a megakaryocyte cell line (Saito, 1997). More importantly, megakaryocyte differentiation can only be induced with physiologically irrelevant substances such as phorbol ester 12-myristate 13-acetate (PMA) (Saito, 1997). The major breakthrough in megakaryocyte research came after the cloning and purification of thrombopoietin (TPO) which enabled researchers to produce megakaryocytes near purity *in vitro* (Majka et al., 2001). Additionally, replacing fetal bovine serum with serum substitutes (containing

bovine serum albumin, human transferrin and recombinant human insulin, StemCell Technologies, 2008) allowed avoiding the inhibitory effects of substances such as transforming growth factor- β1 in serum and further contributed to the success of *in vitro* megakaryocyte production (Ishibashi et al., 1987, Majka et al., 2001, Chen et al., 2009).

Even though there have been major improvements with *in vitro* megakaryocyte production, there are great differences in megakaryocyte culture conditions among studies which can affect the outcome of cultures (yield, maturation and time course of cultures). The main culture conditions affecting the outcome of megakaryocyte cultures are: the source of HPCs, the concentration of TPO, combination of cytokines, growth factors and their concentration. Table 1 summarizes megakaryocyte culture conditions from several studies and the differences between them. This table shows the variations in megakaryocytes culture conditions such as sources of HPCs and TPO concentrations.

1.2.1 Sources of HPCs:

Human HPCs can be obtained from several sources. There are a few differences in HPCs from different sources in terms of their ability to produce megakaryocytes; for example, it takes a longer time for HPCs from cord blood (CB-HPCs) to become megakaryocytes whereas HPCs from peripheral blood and bone marrow (PB-HPCs and BM-HPCs) take less time and produce more mature megakaryocytes (Schipper et al., 2003). The characteristics and function

of HPCs in megakaryocyte cultures from different sources have been thoroughly explained in section 1.3.

1.2.2 TPO and other cytokines in megakaryocyte culture:

TPO, as the main regulator of megakaryocyte and platelet production, is the major component of a megakaryocyte culture; however, the concentration of TPO varies significantly among studies (5-100 ng/mL) (Table 1). The use of other growth factors and cytokines at various concentrations in addition to TPO can also affect the outcome of the megakaryocyte culture. Interlukin-3 (IL-3) has been used in some cultures to increases the proliferation of megakaryocytes; however, it has also been shown IL-3 suppresses megakaryocyte maturation (Dolzhanskiy et al., 1997, 1998). Other cytokines such as IL-11, Fms-like tyrosine kinase 3 ligand (Flt-3L) and stem cell factor (SCF) have megakaryopoietic properties which, in synergy with TPO, can significantly increase the yield and maturation of megakaryocytes (Broudy et al., 1995, Drayer et al., 2000, De Bruyn et al., 2005). A large variation in TPO concentration and the use of different growth factors in various concentrations are one of the main discrepancies in studies which use megakaryocyte cultures as a bioassay.

1.2.4 Megakaryocyte cultures for studies of immune thrombocytopenia:

The recent advancements in development of megakaryocyte cultures have enabled researchers with *in vitro* investigation of platelet production

disorders such as immune thrombocytopenia (ITP), *MYH9*-related thrombocytopenia and myeloproliferative neoplasms (Chang et al., 2003, Pecci et al., 2009, Vicari et al., 2012, Balduini et al., 2011). Table 1 also summarizes various investigations of platelet disorders using megakaryocyte cultures. Here, investigation of platelet production in ITP using megakaryocyte cultures has been discussed.

ITP is an autoimmune disease characterized by a low peripheral platelet count (thrombocytopenia) in the absence of any other causes that lead to a decreased platelet count (Provan et al., 2010). Early studies in identifying the pathogenic cause of ITP resulted in identification of antiplatelet antibodies of IgG class mainly against CD41/61and CD42 (McMillan, 2000). In ITP, antibody coated platelets are sequestered to the reticuloendothelial system and destroyed by macrophages (Luck et al., 1980). There are numerous lines of evidence from experimental and clinical studies which suggest that platelet production is also impaired in a group of ITP patients (McMillan et al., 1978, Damesk and Miller, 1946, Houwerzijl et al., 2004, Louwes et al., 1999). It has been shown that antiplatelet antibodies from ITP patients can bind to megakaryocytes which may impair their normal function (McMillan et al., 1978). Ultra-structural and morphological examinations of bone marrow biopsies from ITP patients have shown a shift to less mature megakaryocytes along with the presence of abnormal features which may indicate megakaryocytic injury (Damesk and Miller, 1946, Houwerzijl et al., 2004). These features disappeared when ITP patients

received therapies and went into remission (Damesk and Miller, 1946, Houwerzijl et al., 2004). In recent years, a few studies have shown that the addition of plasma or purified IgG from ITP patients in a megakaryocyte culture can affect the normal growth of megakaryocytes (Chang et al., 2003, McMillan et al., 2004, Yang et al., 2010); however; the differences in culture conditions among these studies have led to significant differences in their results (Table 1). The megakaryocyte cultures by Chang et al. had shown a significant suppression of megakaryocytes by a group of ITP sera; however, this megakaryocyte culture only received a low dose of TPO (10 ng/mL) for 8 days which lead to a low yield (less than 20%) and low maturation of megakaryocytes with polyploidy distribution of only 2N and 4N (Chang et al., 2003). McMillan et al. also used a megakaryocyte culture for investigating the suppressive effects of ITP plasma/IgG on normal megakaryopoiesis and their result agree with Chang et al. Due to using PB-HPCs, megakaryocytes had higher polyploidy (up to 16N); however, the TPO concentration at 5 ng/mL could only produce a megakaryocytic yield of 50% in this culture (McMillan et al., 2004). Contradictory to Chang et al. and McMillan et al., Yang et al. showed that the presence of ITP sera in megakaryocyte cultures didn't suppress the proliferation of megakaryocytes but it interfere with their maturation; however, their megakaryocyte cultures contained a significantly higher concentration of TPO (100 ng/mL) and SCF (100 ng/mL) (Yang et al., 2010). It has been shown that SCF increases megakaryopoiesis in presence of TPO and can protect megakaryocytes from inhibitory effects of

cytotoxic agents in the culture (Zeuner et al., 2007); therefore, high concentration of SCF and TPO may mask the suppressive effects of ITP plasma/antibodies. In other words, if the concentration of TPO in a culture is not optimized, the results from that culture will not be reliable for investigation of suppressive effects of ITP antibodies on normal megakaryopoiesis. Therefore, the establishment of TPO-dependent dose response in megakaryopoiesis is important in order to find an optimal TPO concentration which supports the production of mature and functional megakaryocytes and concurrently allows the identification of the suppressive effects of ITP antibodies.

1.3 Hematopoietic progenitor stem cells (HPCs):

Hematopoietic progenitor cells (HPCs) play an important role in blood regeneration with the ability to differentiate into all types of blood cells. HPCs reside in bone marrow and are identified by the surface marker, CD34+ which is a glycosylated transmembrane adhesion protein with a possible role in the homing of HPCs in the bone marrow (Wognum et al., 2003). The extracellular domain of CD34 molecule has three class epitopes (class I, II, III) based on the sensitivity to enzymatic cleavage with glycoprotease from *Pasteurella haemolytica* and neuraminidase (Sutherland, 1992). Class III epitope is abundantly expressed compared to the other two class epitopes; Hence, the majority of monoclonal antibodies used for identification and isolation of HPCs are raised against the class III epitope (Egeland, 1998). In addition, CD34 is also

expressed in low numbers on small vessel endothelial cells and a subset of fibroblasts and in high numbers in acute myeloid leukemic cells (Lanza et al., 1999, Gerber et al., 2012). HPCs are found in the mononuclear cell (MNC) fraction of white blood cells and have approximately the same size and granularity as lymphocytes (Barnett et al., 1999).

1.3.1 Sources of HPCs: bone marrow, cord blood, mobilized and normal peripheral blood:

HPCs come from variety of sources with differences in numbers, proliferative and maturation capabilities. These differences affect the decision of researchers in selecting a source of HPCs for *in vitro* studies. There are four sources of HPCs: bone marrow, cord blood, mobilized peripheral blood and normal peripheral blood.

Bone marrow contains the highest number of HPCs cells with a frequency range of 5.6 ± 4.6% of all mononuclear cells (MNCs) (Fritsch et al., 1995); however, difficulties associated with the acquisition of bone marrow samples outweighs its richness for HPCs. The collection of HPCs via bone marrow biopsy is highly invasive, requires local anesthesia, yields only 1-2 mL of marrow from a single biopsy and is exclusively performed by a hematologist (Rodak et al., 2007); therefore, due to lack of availability and highly invasive and complicated sample collection procedure, the bone marrow is not an ideal source of HPCs.

Mobilized peripheral blood is also a rich source of HPCs with a frequency range of 1.9± 2.6% of MNCs (Fritsch et al., 1995,). Once a mobilizing cytokine such as granulocyte colony stimulating factor (G-CSF) is administered, HPCs are mobilized from bone marrow into peripheral blood raising the frequency of HPCs (Motabi et al., 2012). The mobilized cells can be harvested by collecting a large number of white blood cells from circulation thereby making it suitable for studies requiring high yield of HPCs (Motabi et al., 2012). Even though HPCs mobilization can be performed in healthy volunteers, the primary aim for this procedure is to provide patients receiving chemotherapy with autologous HPCs transplantation (Motabi et al., 2012); hence, the availability of these cells for *in vitro* research is limited.

Cord blood (CB) is another rich source of HPCs with a frequency range of 1.7± 2.6% of MNCs (Fritsch et al., 1995). CB-HPCs are harvested from the umbilical cord blood obtained from the fetal side of a full term placenta (Mehrishi and Bakács, 2013). Because the acquisition of umbilical cord blood is not invasive and large volume of cord blood can be obtained, CB-HPCs are widely used for *in vitro* studies (Table 1); however, with the increasing use of cord blood for hematopoietic stem cell transplantation and the availability of this source only to researchers with access and affiliation to clinical settings, cord blood is not universally available (Oran and Shppall, 2012).In the steady state, HPCs are present in small numbers in peripheral blood. The origin of PB-HPCs is the bone marrow where HPCs are mobilized and then enter the circulation in order to

participate in tissue regeneration of small vessels (Crosby et al., 2000). Peripheral blood has the lowest frequency of HPCs. On average, HPCs consist of 0.076 ± 0.057% of all MNCs in peripheral blood; however, the frequency of HPCs can significantly vary (Cohen et al., 2013). HPC frequency has been inversely correlated with older age, female gender and smoking (Cohen et al., 2013). Additionally, since the frequency of HPCs is highly heritable, genetic variation also contributes to variations in the frequency of HPCs among different individuals (Cohen et al., 2013). Peripheral blood is obtained by vein puncture which is less invasive compared to bone marrow samples and the donation of 450 mL of venous blood (with fifty six days waiting period before the next donation) can be tolerated by healthy donors without any major adverse effect (Harmening, 2005). Peripheral blood is the only source of HPCs which is universally available and accessible by all investigators and despite the low frequency of HPCs in peripheral blood, it has been used in numerous *in vitro* studies (Table 1).

1.3.2 The use of HPCs in Megakaryocyte cultures:

Megakaryocytes have been grown *in vitro* from all sources of HPCs; however, the quality of cultured megakaryocytes and the kinetics of megakaryopoiesis vary according to the type of HPCs (Schipper et al., 2003, Yasui et al., 2003). It has been shown that cord blood HPCs have higher proliferative capabilities in response to TPO; however they show delayed

maturation compared to BM-HPCs and PB-HPCs (Schipper et al., 2003, Mattia et al., 2009). Several studies have confirmed that megakaryocytes from BM-HPCs and PB-HPCs show higher polyploidy level (8N and higher), while megakaryocytes from CB-HPCs have reduced polyploidization (Mattia et al., 2009, Leysi-Derilou et al., 2010, Debili et al., 1995, Guerriero et al., 1995).

The difference in megakaryopoiesis has been related to differences in phenotypes and subsets of HPCs (Yasui et al., 2003). Myeloid progenitors from which megakaryocyte progenitors are produced are present in a higher percentage in PB-HPCs compared to BM-HPCs (Deutsch et al., 2013, Fritsch et al., 1995).

CD133 is an early hematopoietic marker which co-expresses with CD34 on HPCs and it has been shown that HPCs with the phenotype of CD34+CD133- have a higher ability to differentiate and produce mature megakaryocytes (Yasui et al., 2003). PB-HPCs contains a higher percentage of CD34+CD133- cells compared to cord blood, thereby making peripheral blood the better source for megakaryopoiesis in terms of producing more mature megakaryocytes (Yasui et al., 2003).

1.3.3 Advantages and limitations associated with the use of HPCs from normal peripheral blood for megakaryocyte cultures:

As mentioned in 1.3.1 and 1.3.2 sections, the use of peripheral blood as a source of HPCs for megakaryocyte cultures has several advantages. A major limitation associated with the use of PB-HPCs is the low frequency of HPCs resulting in a low isolation yield which makes PB-HPCs an inefficient source of HPCs for large scale experiments.

In addition to the low isolation yield, while the ideal purity of HPCs after purification is 90% or over, the low frequency of HPCs in peripheral blood leads to lower isolation purity. Some studies which used PB-HPCs for megakaryocyte cultures used only samples with isolation purity over 90% from selected donors (Table 1). There are also small numbers of studies which either didn't report the purity of PB-HPC isolations or they went ahead and used samples with low purity (McMillan, 2004, Balduini et al., 2009).

Author	Source of HPCs	Purity of HPCs	TPO (ng/mL)	Other growth factors/ cytokines (ng/mL)	Duration of cell culture (days)	Disease under investigation
Carpnell et al. (2000)	BM	70-90%	10 U/mL	SCF: 200 IL-3: 50 Flt-3: 50	14	CMV-ITP
Lepage et al. (2000)	CB	> 90%	50	IL-6: 10 IL-11: 10 IL-3: 2.5	14	MK biology
Chang et al. (2003)	CB	ND	10	None	8	ITP
Yang et al. (2010)	CB	> 90%	100	SCF: 100 IL-6, IL-3: 10	8 to 15	ITP
Houwerzijl et al. (2004)	MPB	> 90%	20	SCF: 10	7 and 12	ITP
Perdomo et al. (2011)	MPB	ND	50	None	12	QITP
McMillan et al. (2004)	PB	30-35%	5	None	10	ITP
Balduini et al. (2009)	PB, CB	ND	10	IL-11: 10 IL-6: 10	12	BSS
Veljkovic et al. (2009)	PB	> 90% from selected donors	50	None	13	QPD
Pecci et al. (2009)	PB	ND	10	IL-11: 10 IL-6: 10	14	MYH9-RD
Balduini et al. (2009)	PB	ND	10	IL-3: 10 IL-6: 10	14	MPN
Nurden et al. (2010)	PB	> 95% From selected donors	100	SCF: 40 IL-3: 3	7 or 14	VWD2B

Table 1: A Summary of megakaryocyte cultures and sources of HPCs in the literature.

Abbreviations: CMV-ITP (cytomegalovirus induced thrombocytopenia), MK (megakaryocyte), ITP (immune thrombocytopenia), MPB (mobilized peripheral blood), QITP (Quinine -induced immune thrombocytopenia), ND (not determined), BSS (Bernard-Soulier syndrome), QPD (Quebec platelet disorder), *MYH9*-RD (*MYH9* Related

Disease), MPN (Myeloproliferative Neoplasm), VWD2B:Von Willebrand disease type 2B, PB (peripheral blood), CB (cord blood), BM (bone marrow), Flt-3 (Fms-like tyrosine kinase 3), IL-3, 6, 11 (Interleukin 3, 6, 11), SCF (stem cell factor).

1.4 Rationale and hypothesis:

Despite the value of using PB-HPCs cells in megakaryocyte cultures, no study has developed a culturing system to utilize PB-HPCs in an efficient manner. More importantly, it is not known whether purified PB-HPC samples with purities lower than 90% can successfully produce highly pure megakaryocyte cultures. The minimum isolation purity of HPC samples which can successfully produce megakaryocytes is also not known.

Additionally, there is a need for an optimized megakaryocyte culture for the investigation of ITP in a way that mature megakaryocytes with high purity is produced without masking the suppressive effects of ITP antibodies/plasma on normal megakaryopoiesis. This requires the presence of a megakaryopoiesis-TPO dose response to establish an optimal TPO concentration suitable for such studies.

In this study, we developed a megakaryocyte culturing method from human peripheral blood CD34+ cells as the most accessible source of HPCs which can produce highly pure and mature megakaryocytic cells. This method is also suitable for large scale experiments and TPO-dose dependent for the investigation of ITP antibodies/plasma on normal megakaryopoiesis.

Hypothesis:

PB-CD34+ cell samples with isolation purity lower than 90% can be utilized with high efficiency to produce mature (polyploid) and functional megakaryocyte in a TPO dose dependent manner.

CHAPTER 2: METHODS

2.1 Reagents and buffer preparation:

2.1.1 CD34+ wash buffer:

This buffer contained 1% (w/v) bovine serum albumin (BSA), 2mM ethylenediaminetetraacetic acid (EDTA), 1mM theophylline, 0.02mM Prostaglandin E1 (PGE1), 0.154µM aprotinin in magnesium and calcium free phosphate buffer solution (PBS). The buffer was prepared at the pH of 7.4 at the room temperature and filter sterilized with a 0.2µm filter before use. This buffer can be stored at 4ºC for one week.

2.1.2 Flow cytometry wash buffer:

This buffer contained 0.5% (w/v) BSA and 2mM EDTA in magnesium and calcium free PBS. The solution was filtered with a 0.4µm filter to remove the aggregates and undissolved BSA.

2.1.3 OptiPrep™ diluent solution and OptiPrep™ working solution:

The diluent solution contained 145mM sodium chloride, 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1mM EDTA, pH7.4 (Graham, 2002). The diluent solution was mixed with OptiPrep™ (Axis Shield) in a 1:1 ratio in order to achieve the density barrier of ρ = 1.063 g/mL (Graham, 2002). The diluent solution and the Optiprep were mixed just before use.

2.1.4 Anticoagulant:

Acid citrate dextrose (ACD) was used as the anti-coagulant and contained 85.00mM sodium citrate (tri-sodium citrate), 66.62mM citric acid (monohydrate) and 111.01mM dextrose. The pH was adjusted to 4.5 and the solution filter sterilized with a 0.2µm filter. The ratio of anticoagulant to blood is 1:6 (one part ACD, 6 parts blood).

2.1.5 Paraformaldehyde fixative solution:

8% (w/v) Paraformaldehyde (PFA) was prepared in magnesium and calcium free PBS at 70°C. After the solution turned clear, it was filtered with a 0.4µm filter to remove aggregates and undissolved PFA. Immediately before use sample were diluted 1:1 with the 8% PFA solution (working solution of PFA was 4% w/v).

2.1.6 Complete growth medium:

The growth media was Iscove's Modified Dulbecco's Medium (IMDM) containing GlutaMax^{TM}, 25mM HEPES, 0.0399mM phenol red and 1mM sodium pyruvate (Gibco®). The media was supplemented with 1% penicillin/streptomycin (Gibco®) and 15% serum substitutes (BIT9500, Stemcell Technologies). The serum substitute contains BSA, insulin and human transferrin (iron-saturated) (Stemcell Technologies, 2008)

2.1.7 Propidium iodide (PI) staining solution:

The buffer contains 100mM Tris (pH 7.4), 150mM sodium chloride, 1mM calcium chloride, 0.5mM magnesium chloride, 0.05% Triton X-100 (Invitrogen, 2006). The buffer was filter sterilized with a 0.2µm filter.

2.1.8 Cytokines and antibodies:

All cytokines were purchased from R&D Systems, Inc. Lyophilized cytokines were re-suspended in sterile, 0.2% (v/v) fetal bovine serum (Gibco®) in PBS. All flow cytometry antibodies were purchased from BD Biosciences, except Raj-1 (mouse monoclonal anti-human CD41/61) which was produced in our laboratory.

2.2 Isolation of CD34+ cells from peripheral blood:

2.2.1 Blood Samples:

90mL or 180mL of whole, venous blood was drawn after obtaining informed consent from healthy donors with no history of platelet disorder. All venipuncture punctures were performed by certified medical laboratory technologists. Blood was collected in 50mL sterile tubes containing ACD (30mL of blood to 5mL of ACD). The tubes and the anticoagulant were brought to room temperature in order to avoid any activation of platelets in the plasma. The blood was mixed with the ACD by gently inverting the tubes three to five times. In order to avoid contamination, the caps of the sterile tubes were opened just before the

addition of blood and were closed immediately afterward. All the procedures thereafter were performed inside a level II biosafety cabinet.

2.2.2 Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood:

Tubes containing anticoagulated whole blood were centrifuged at 170*g* for 30 minutes (min) without braking at room temperature. The top 2/3 of the platelet rich plasma (PRP) layer was gently removed without disturbing the cellular part of the blood. Each tube contained approximately 20-25mL of blood components after PRP removal. CD34 wash buffer was added to each tube to bring the total volume to 35mL. In a separate sterile 50mL tubes, 15mL of Histopaque (Sigma Aldrich) was added. The diluted blood samples were layered onto the Histopaque, very slowly and carefully. Tubes containing the blood sample and Histopaque were centrifuged at 750*g* for 30min without braking at room temperature. The top 3/4 of the PRP was removed from each tube and PBMCs were collected from the buffer/Histopaque interface and added to new sterile 50mL tubes (up to 25mL in each tube). Each tube was filled up to 50mL with CD34 wash buffer and centrifuged at 370*g* for 10min with braking at room temperature. The supernatants were discarded and the pellets were resuspended and pooled into one 50mL tube. The volume was adjusted to 25mL using the CD34 wash buffer. In order to deplete the platelets, a solution of Optiprep was used as described by Graham et al, 2002. PBMCs were gently layered onto 25mL of the working Optiprep solution and the tube was centrifuged at 350*g* for

15min without braking at room temperature. The supernatant was discarded and PBMCs were resuspended in CD34+ wash buffer. In order to further deplete platelets, the tube was centrifuged at a lower speed (200*g* for 12min with braking). The supernatant, containing platelets, was discarded. The pellet was resuspended in 500µL of CD34+ wash buffer and the volume of the cell suspension was measured and recorded.

2.2.3 Isolation of CD34+ cells from PBMCs:

CD34+ cells were isolated by modifying methods described by Stemcell Technologies, 2009 and Miltenyi Biotech, 2007. A sample of 1:20 dilution of PBMCs was prepared in a 1.5mL Eppendorf tube. This sample was used to enumerate the total number of PBMCs. The total number of white blood cells was counted by using an automated counter (Coulter A^c .T diff, Beckman Coulter) and multiplied by the dilution factor (20). The cell concentration was adjusted to 2 x 10^8 cells/mL with the CD34+ wash buffer. For every 1mL of the cell suspension, 100µL of the CD34+ selection cocktail (StemSep® human CD34+ positive selection kit, StemCell Technologies) was added and the cell suspension was incubated at 4°C for 10min with the selection cocktail. For every 1mL of the cell suspension, 60µL of the dextran-coated magnetic colloid bead suspension (StemSep® human CD34+ positive selection kit, StemCell Technologies) was added and the cell suspension was incubated at 4°C for 10min with the magnetic beads. The anti-CD34 antibody is complexed with anti-dextran forming bispecific

tetrameric antibody complexes which recognize both dextran colloid beads and the target cell surface antigen (StemCell Technologies, 2009). The cell suspension was washed with the 20mL of CD34+ wash buffer (370*g* for 10min with braking). The supernatant was discarded and the pellet was resuspended with 25-30mL of CD34+ wash buffer. The cells were passed through a selection column (LS columns, Miltenyi Biotech) placed in a magnet (QuadroMACS™ Separator, Miltenyi Biotech). CD34+ cells labeled with magnetic beads were then separated from unlabeled cells by passing them through the magnetic separation column (StemCell Technologies, 2009). Captured cells were washed three times with 3mL aliquots of the wash buffer and the column was then removed from the magnet and placed over a clean sterile 15mL tube. The cells were eluted with 5mL of CD34 wash buffer into the tube and gently mixed. In order to further purify CD34+ cells, eluted cells were passed through another selection column as described earlier. Eluted cells were collected in a new sterile 15mL tube and centrifuged at 370*g* for 10min at the room temperature. The supernatant was removed and cells were resuspended in 5mL of complete growth media.

2.3 Megakaryocyte cell culture:

All cell cultures were incubated at 37°C and 5.0% CO_2.

2.3.1 Megakaryocyte cell culture with *ex vivo* expanded CD34+ cells:

a) *Ex vivo* expansion of CD34+ cells:

The *ex vivo* expansion of CD34+ cells were carried out by modifying a method described by Invitrogen, 2001. CD34+ cells were isolated from 90mL of whole blood. In order to expand CD34+ cells *ex vivo*, isolated cells were seeded at the cell concentration of 3-5 x 10^4 cells/mL (5mL of the cell suspension per well) in 6-well tissue-culture plates and expanded for five days in the presence of IL-3 (50 ng/mL), IL-6 (10 ng/mL), stem cell factor (SCF, 100 ng/mL) and granulocyte-monocyte colony stimulating factor (GM-CSF, 10 ng/mL) (Life Technologies, 2012).

b) Megakaryocyte culture with *ex vivo* expanded CD34+ cells:

Ex vivo expanded CD34+ cells were washed with the complete growth media (5mL of complete growth media and 5mL of cell suspension) by centrifugation at 370*g* for 10min with braking at the room temperature. Washed cells were resuspended with 5mL of complete growth media and CD34+ cells were enumerated and seeded at 5 x 10^4 CD34+ cells/well (1mL of the cell suspension per well) in 24-well tissue-culture plates. Cultures were set up in the presence of recombinant human thrombopoietin (TPO) at various concentrations for 8-13 days and TPO was added to cultures every four days. On day 8, half of the media was removed and substituted with the same amount of fresh media.

2.3.2 Micro volume megakaryocyte cell cultures:

CD34+ cells were isolated from 180mL of peripheral blood and were seeded at 1 x 10^4 CD34+ cells/well in a total volume of 0.2mL in flat bottom 96-

well tissue-culture plates and cultured in the presence of various concentrations of TPO (0-50 ng/mL) for 11 to 16 days. In order to prevent evaporation of media during the long incubation period, all the empty wells surrounding the wells containing the cultured cells were filled with PBS. Cultures were supplemented with TPO at the same concentration as day 0 (20 ng/mL) every four days.

2.4 Total cell enumeration:

Cells were mixed with trypan blue in a 1:1 ratio and added to the counting chambers (Countess® Cell Counting Chamber Slides, Life Technologies). Cells were counted with a Countess® automated cell counter (Life Technologies) in order to determine the viability and total number of viable cells. A negative control was included with every batch by adding only trypan blue to a counting chamber and performing a count. This is to ensure a low contribution from the trypan blue.

2.5 Flow cytometry Analysis:

A Beckman Coulter Epics XL with 488nm argon excitation laser was used. Flow Jo (version 7.6.5) was used for data analysis of flow cytometry experiments.

2.5.1 Immunophenotyping

Table 2 lists all the monoclonal anti-human antibodies which were used in flow cytometry experiments.

Antibody	Fluorophore	Reactivity	Working concentration (µg/mL)	Clone	Antibody Class
anti-CD41/61	fluorescein isothiocyanate(FITC)	GPIIb/IIIa	5	Raj-1 (In house)	IgG1
anti-CD42	phycoerythrin (PE)	GPIb	5	HIP1	IgG1
anti-CD34	r-phycoerythrin-cyanine 5 (PE-Cy5)	class III epitope CD34	0.625	581/CD34	IgG1
anti-CD41a	fluorescein isothiocyanate(FITC)	GP IIIa	1.25	HIP8	IgG1

Table 2: Mouse monoclonal anti-human antibodies used in the flow cytometry experiments

In order to validate each batch of in-house anti-CD41/61, isolated human platelets were stained with a commercial monoclonal FITC conjugated anti-CD41 (BD Biosciences) and the in-house FITC conjugated anti-CD41/61 (Raj-1). Both samples were analyzed in parallel by flow cytometry. The flow cytometry voltages for the forward and side scatter settings were adjusted by using unstained isolated PBMCs. The discriminator (threshold) was set to discriminate platelets and red blood cells. Single, double and triple stained cultured cells from day 0, 7, 11 and day 15 were used in order to compensate for the spillover from flourophores. The detailed flow cytometry settings are listed in table 3.

	Voltage	Gain
Forward scatter	140	1.0
Side scatter	275	10.0
FITC	677	1.0
PE	880	1.0
PECy5	980	1.0

Table 3: Flow cytometry settings used for immunophenotyping experiments with the Beckman Coulter Epics/XL flow cytometry instrument.

Immunophenotyping was carried out under the high rate acquisition mode. With the exception of CD34 purity measurement, flow events with high granularity (stretched across the side scatter) and small size were excluded in the analysis. This population was shown to have high auto-fluorescence. The majority of the PI positive events also were in this population.

2.5.2 Flow cytometry controls:

Unstained samples were used to set the background and identify the population with the auto-fluorescence. Fluorophore matched mouse IgG isotype controls (IgG1 κ) were used at the same concentration as their corresponding test antibodies in all flow cytometry experiments. Isotype controls were used to show the amount of non-specific staining of flourophores in order to determine the true positive flow cytometry events.

2.5.3 Flow cytometry immunostaining:

Cell suspensions were transferred to 1.5mL Eppendorf tubes. All antibodies were added to 100µL of cell suspension having < 1 x 10^6 cells in total. Cell suspensions were gently mixed and incubated at 4°C for 20min in the dark. After cells were brought to room temperature (incubating the tubes at room temperature for 10min) 500µL of flow cytometry buffer was added and analyzed.

2.5.4 Flow cytometry immunostaining of micro volume cultures:

The contents of each well (200µL) were transferred to a 1.5mL Eppendorf tubes and wells washed with PBS (50µL). The 50µL of PBS from each well was then transferred to the Eppendorf tube and gently mixed. For each triplicate set, 50µL from each tube was transferred to a 1.5mL Eppendorf tube (150µL) and gently mixed. This tube was used for the IgG isotype control for the whole triplicate set.

2.5.5 DNA content assessment:

Cultured cells were transferred into a 1.5mL Eppendorf tube, centrifuged at 300*g* for 10min and the supernatant removed. Cells were resuspended in PBS (1mL), centrifuged and the supernatant was discarded. The pellet was resuspended in PBS (100µL). Subsequently, the tube was placed on a mixer at a low speed and –20°C methanol (Caldeon) was added up to 1mL. Cells were fixed for 30min in methanol at 4°C or on ice with occasional gentle mixings. After

30min, cells were centrifuged at 400*g* for 10min, the supernatant was removed and PBS (1mL) was added quickly. The pellet was thoroughly resuspended and incubated in PBS for 15min in order to rehydrate the cells. Fixed cells were centrifuged at 400*g* for 10min and washed with PBS. PI staining buffer (400µL) was added to the cell pellet and cells were resuspended. DNA free RNAase (Sigma Aldrich) was added to a final concentration of 100 µg/mL and cells were incubated at 37°C for 30min. After the incubation, PI (Sigma Aldrich) was added (20 µg/mL) and cells were incubated for 15min at room temperature in the dark. After the incubation, 500µL of PI staining buffer was added to the tube. The cell suspension was transferred to a polystyrene tube and the stained cells were analyzed by flow cytometry. In order to locate the position of 2N peak, PBMCs (which mainly contain 2N cells) were used as a control by fixing, staining and analyzing them in the same manner. The flow cytometry settings are listed in table 4. The discriminator (threshold) was set to discriminate the particles and cells at the same size of red blood cells and platelets. DNA content assessment was carried out under the low rate acquisition mode.

	Voltage	Gain
Forward scatter	140	1.0
Side scatter	275	5.0
PI	450	1.0

Table4: Flow cytometry settings used for DNA content (Polyploidy) assessment with the Beckman Coulter Epics/XL flow cytometry instrument.

2.6 Microscopy

2.6.1 Live cell microscopy:

Cultures in flat bottomed tissue-culture plates were viewed on an inverted bright field microscope (CKX41, Olympus) equipped with a camera (Infinity1-3C, Lumenera Corporation). Images of cells at 10X and 40X magnifications were captured. ImageJ (version 1.47) was used to set the scale bars.

2.6.2 Hematoxylin and eosin (H and E) staining:

Cultured cells were centrifuged using a cytospin(Cytospin 3, Shandon) at 300rpm on to glass slides (Surgipath Apex, Lecia microsystem) coated with albumin as an adhesive reagent. Cells were subsequently fixed and stained with H and E as follows:

1) Fixative solution (Hema-Diff Fixative, Nova Ultra), 30 seconds

2) Eosin, 45 seconds

3) Water, 8 dips

4) Hematoxylin, 15 seconds

5) Water, 8 dips

After the slides were dried, one to two drops of a resin based mounting medium was placed on each slide, covered with a glass coverslip and examined with the bright field microscope (CKX41, Olympus). Images were captured with the camera (Infinity1-3C, Lumenera Corporation).

2.6.3 Immunofluorescence staining and confocal microscopy:

Cells were collected in a 1.5mL Eppendorf tube and washed once with PBS (300*g,* 10min). The supernatant was removed and cells were resuspended in 500 µL of PBS. An equal volume of 8% PFA was added to make the final 4% PFA concentration and cells were fixed for 15min in the dark at room temperature. After the PFA fixation, cells were washed twice with PBS, resuspended in PBS (100-200µL) and centrifuged (Cytospin 3, Shandon) at 300rpm onto glass slides (Surgipath Apex, Lecia microsystem). Immediately after centrifugation, slides were transferred to a wet chamber and 100µL of PBS was added on each slide in order to prevent the cells from drying. The immunostaining of cells was carried out as follows:

1) Permeabilization: 0.05% Triton X-100 in PBS for 5min at the room temperature
2) Wash: twice with PBS
3) Blocking: 3% BSA for 30min at the room temperature
4) Wash: once with 0.5% BSA
5) Indirect immunostaining: Primary mouse anti-human CD42 (5 µg/mL in 3% BSA) for 1 hour at the room temperature following 3 washes with 0.5% BSA, secondary tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC) conjugated goat anti-mouse (Abcam, 1 µg/mL in 3% BSA) for 1 hour at the room temperature in dark.
6) Wash: three times with 0.5% BSA

7) Direct staining: FITC conjugated mouse anti-human CD41/61 (5 µg/mL in 3% BSA) for 1 hour at room temperature.
8) Wash: twice with 0.5% BSA and once with distilled water.
9) Nucleus staining and mounting medium: 2 drops of Fluoroshield™ with DAPI (Sigma-aldrich, an aqueous based mounting medium containing an anti-fading reagent and 4',6-diamidino-2-phenylindole, DAPI) were added to each slide and incubated at room temperature for 5min in the dark . Slides were covered with glass coverslips and sealed.

Control slides were prepared to show the background and nonspecific staining in the same manner as test slides except: in step 5 of immunostaining, 3% BSA was added instead of anti-human CD42 and in step 7, FITC conjugated mouse IgG isotype control was added at 5 µg/mL instead of FITC conjugated mouse anti-human CD41/61. Immunofluorescence confocal microscopy was performed by the Leica MP TCS-SP5 microscope at McMaster Biophotonics facility. FITC conjugated mouse anti-human CD41/61 and the primary mouse anti-human CD42 are in-house antibodies.

2.7 Measurement endpoints:

2.7.1 CD34+ cell isolation:

a) CD34+ cell Purity:

The CD34+ cell purity was defined by the percentage of flow cytometry events with positive CD34 and low granularity (the same granularity as peripheral blood lymphocytes).

b) Megakaryocyte purity:

The megakaryocytic cell purity was defined by the percentage of all flow cytometry events with positive CD41/61.

c) CD34+ cell Isolation yield:

The yield of CD34+ cell isolation was calculated by multiplying the purity of CD34+ cells (measured by flow cytometry) by the total number of live cells (measured by trypan blue exclusion).

2.7.2 Viability:

The viability was determined by trypan blue exclusion from total number of live cells divided by total number of cells times 100.

2.7.3 Total number of megakaryocytes (megakaryocyte growth):

Total numbers of megakaryocytes were calculated by multiplying the percentage of positive CD41/61 events by the total number of live cells.

2.7.4 Total number of mature megakaryocytes:

Total numbers of mature megakaryocytes were calculated by multiplying the percentage of CD41/61+ CD42+ by the total number of live cells.

2.7.5 Morphological evaluation:

In live microscopy examination, the morphology of the cell and proplatelets were investigated by using bright field microscopy. Proplatelets were defined as elongated pseudopod-like cells. In H and E stained slides; megakaryocytes were defined as large polyploid cells.

2.7.6 Polyploidy distribution analysis:

The intensity of PI staining was used as a measure of DNA content. The location of the (2N) peak on the PI histogram was determined by the location of PBMC's 2N as the control. The polyploidy was defined as any DNA content ≥4N.

2.8 Statistical analysis:

GraphPad Prism (version 5) was used for plotting graphs and statistical analysis. The presence of a statistical significance was investigated as follows:

- Mann-Whitney test was used to compare the two groups with pooled experimental data from different PB-CD34+ cell donors.
- Student t-test was used to compare the two groups with experimental data from one PB-CD34+ cell donor.
- Multi variant ANOVA was used to compare the more than two groups of experimental data.

CHAPTER 3: RESULTS

3.1 Purity of isolated PB-CD34+ cells:

The average purity of isolated PB-CD34+ cells was 49.2% ± 20.5% (n = 47) with the lowest purity being 10% and the highest 94.1%. Figure 2 illustrates a sample of flow cytometry analysis of purified CD34+ cells.

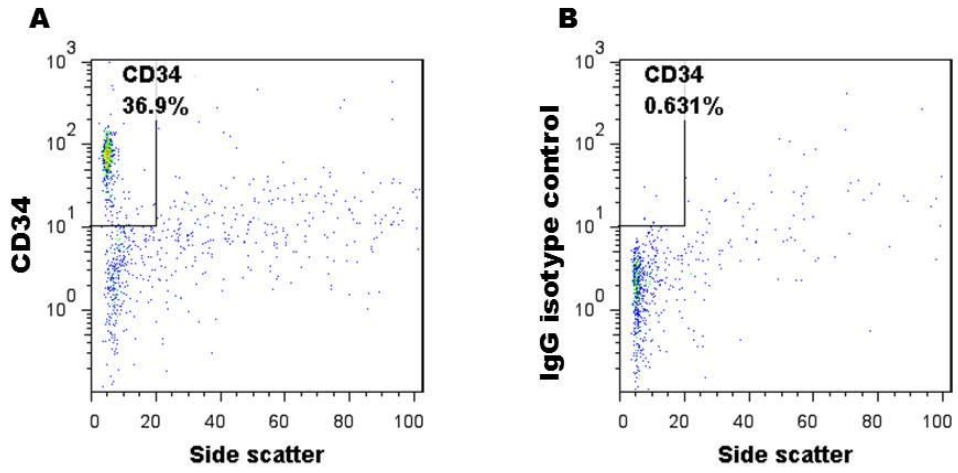


Figure 2. Flow cytometry analysis of isolated CD34+ cells: (A) CD34+ cells were positively isolated from PBMCs and analyzed on flow cytometry by staining with PE-Cy5 conjugated anti-CD34. The purity was expressed as the percentage of CD34+ positive events within purified cells. (B) Minimal nonspecific binding within the gated area is demonstrated by PE-Cy5 conjugated IgG isotype control.

3.2 Megakaryocyte cultures with PB-HPCs in 24-well tissue-culture plates:

The average yield of CD34+ cells from 180mL of PB was $5.5 \times 10^5 \pm 2.5 \times 10^5$ (n = 18). In order to determine the lowest seeding number of CD34+ cells, cultures were set up with 1×10^4, 2×10^4 and 5×10^4 CD34+ cells/well in 1 mL with TPO (10 ng/mL) in 24-well tissue-culture plates (n = 2, different donors). After 11 days of culture, only experiments with the seeding concentration of 5×10^4 CD34+ cells/well were able to produce enough cells which could be reliably enumerated. Therefore, 5×10^4 CD34+ cells/well was used as the seeding number in megakaryocyte cultures with 24-well tissue-culture plates.

In order to investigate megakaryopoiesis-TPO dose response, megakaryocyte cultures were set up in the presence of increasing TPO concentrations (0, 5 and 10 ng/mL) for 11 days. There was a significant difference (p-value = 0.0286) in percentages of cells expressing the megakaryocytic lineage marker, CD41/61, between cultures grown in the presence of TPO (5 and 10 ng/mL) compared to cultures grown in the absence of TPO (figure 3). No correlation was found in CD41/61 expression between TPO 5 and 10 ng/mL.

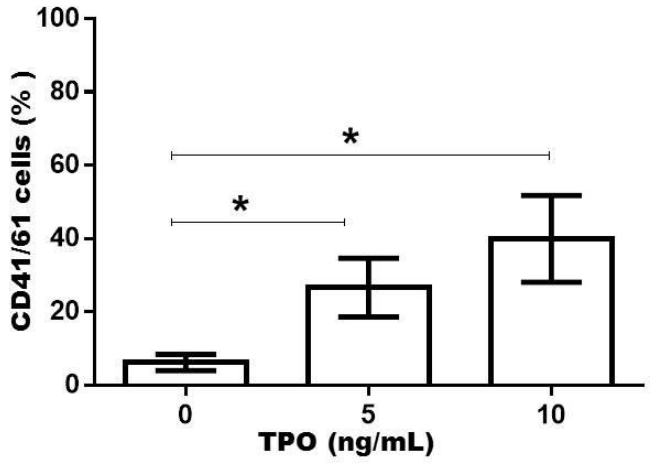


Figure 3. Megakaryocyte differentiation-TPO dose response in 24-well tissue-culture plates: The flow cytometry analysis of cells cultured in the presence of increasing TPO doses for 11 days in 24-well tissue-culture plates showed that there was a significant difference in percentages of differentiated cells (CD41/61+ cells) between cultures grown with TPO at 0 and 5 ng/mL (p-value = 0.0286) and 0 and 10 ng/mL (p-value = 0.0286). Results were pooled from four experiments with different donors. Error bars represent SD. P-values were calculated using Mann-Whitney test. The asterisk, *, represents p-value ≤ 0.05.

Even though this megakaryocyte culture method is TPO dose dependent, the yield of CD34+ cells is insufficient for setting up experiments in an efficient manner. By applying the seeding number of 5 x 10^4 CD34+ cells/well, only three sets of experiments in triplicate can be performed; therefore, an alternative method for *in vitro* production of megakaryocytes was developed.

3.3 Megakaryocyte cultures with *ex vivo* expanded PB-CD34+ cells:

Because of the low yield of isolated PB-HPCs, a two-step culture system was developed: 1) CD34+ cells were expanded *ex vivo* in order to increase the yield of CD34+ cells 2) *ex vivo* expanded CD34+ cells were cultured in the presence of TPO in order to produce megakaryocytes. Purified cells from 90mL of peripheral blood were cultured for five days in the presence of SCF (100 ng/mL), GM-CSF (10 ng/mL), IL-3 (50 ng/mL) and IL-6 (10 ng/mL) in order to increase the number of CD34+ cells. During five days of *ex vivo* expansion, the average fold increase in number of CD34+ cells was 13.4 ± 9.2 with an average purity of 85% (n = 17, different donors). As shown in figure 4, there were significant differences in number of expanded CD34+ cells between day 3 and 4 (p-value = 0.0064), and day 4 and 5 (p-value = 0.0079). No correlation was found between day 2 and day 3. The number of expanded CD34+ cells was significantly higher compared to the number of expanded megakaryocytes (CD41/61+ cells) on day 4 (p-value = 0.0018) and day 5 of expansion (p-value = 0.039) indicating that the combination of cytokines used for *ex vivo* expansion only increased the

number of CD34+ cells and didn't stimulate the cells to differentiate to megakaryocytes (No correlation was found between the number of expanded CD34+ cells and megakaryocytes on day 2 and 3 of expansion).

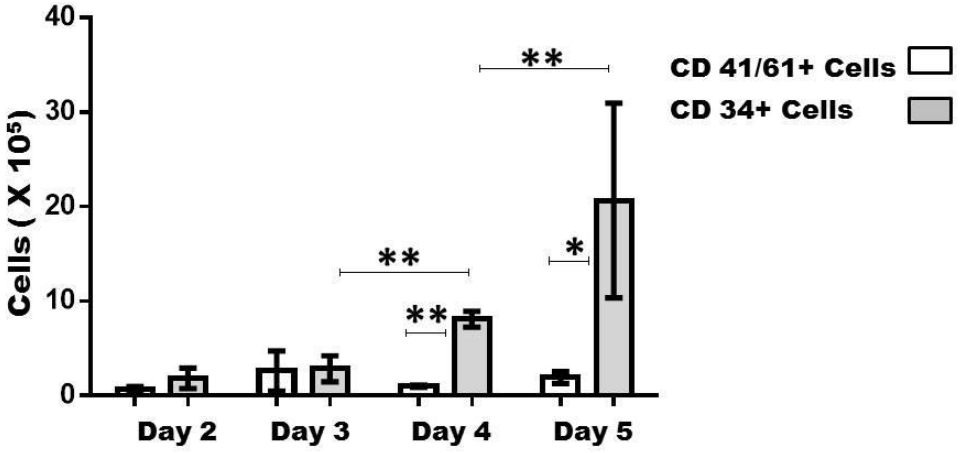


Figure 4. *Ex vivo* expansion of CD34+ cells from peripheral blood: CD34+ cells were isolated from peripheral blood and expanded in presence of SCF, GM-CSF, IL-3 and IL-6. There were significant differences in the number of CD34+ cells between day 3 and 4 (p-value = 0.0064), and day 4 and 5 (p-value = 0.0079). The number of expanded CD34+ cells were significantly higher compared to the number of expanded megakaryocytes (CD41/61+ cells) on day 4 (p-value = 0.0018) and day 5 (p-value = 0.039). Total number of CD34+ cells and megakaryocytes were calculated by multiplying the total number of live cells (measured by trypan blue exclusion) by percentage of flow cytometry CD34+ and CD41/61+ events, respectively. Experiments were set in triplicate from one donor. Error bars represent SD. P-value was calculated by two-tailed Student t-test. The asterisk, *, represents p-value ≤ 0.05 and double asterisks, **, represent p-value ≤ 0.01.

Megakaryocyte cultures set up by seeding 5 x 10^4 *ex vivo* expanded CD34+ cells/well (in 1mL of cell suspension)in the presence of TPO (50 ng/mL) for 8 days produced megakaryocytes with the ability to produce proplatelets (figure 5).

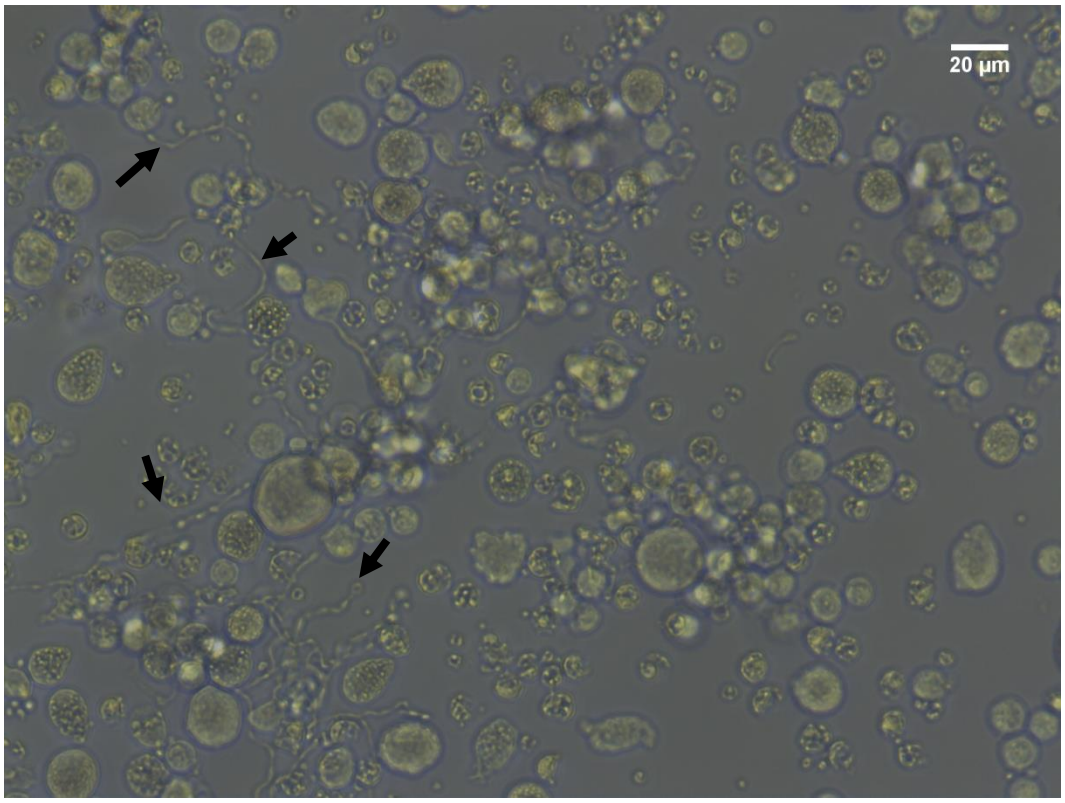


Figure 5. Proplatelet formation in human megakaryocyte cultures with *ex vivo* expanded CD34+ cells: megakaryocyte cultures were set up with *ex vivo* expanded CD34+ cells from peripheral blood in the presence of TPO (50 ng/mL). The live microscopic image shows the formation of proplatelets (black arrows) at day 8 in the megakaryocyte culture.

In order to investigate the megakaryopoiesis-TPO dose response in this megakaryocyte culture system, cultures were set up with *ex vivo* expanded

CD34+ cells in the presence of increasing concentrations of TPO (0, 5, 10, 25 and 100 ng/mL) in 24-well tissue-culture plates and cells were analyzed on day 8, 11 and 13 (n = 2, one donor). No significant difference in megakaryocyte growth was found between cultures at any day of analysis (figure 6), therefore the production of megakaryocytes using *ex vivo* expanded CD34+ cells was independent of TPO.

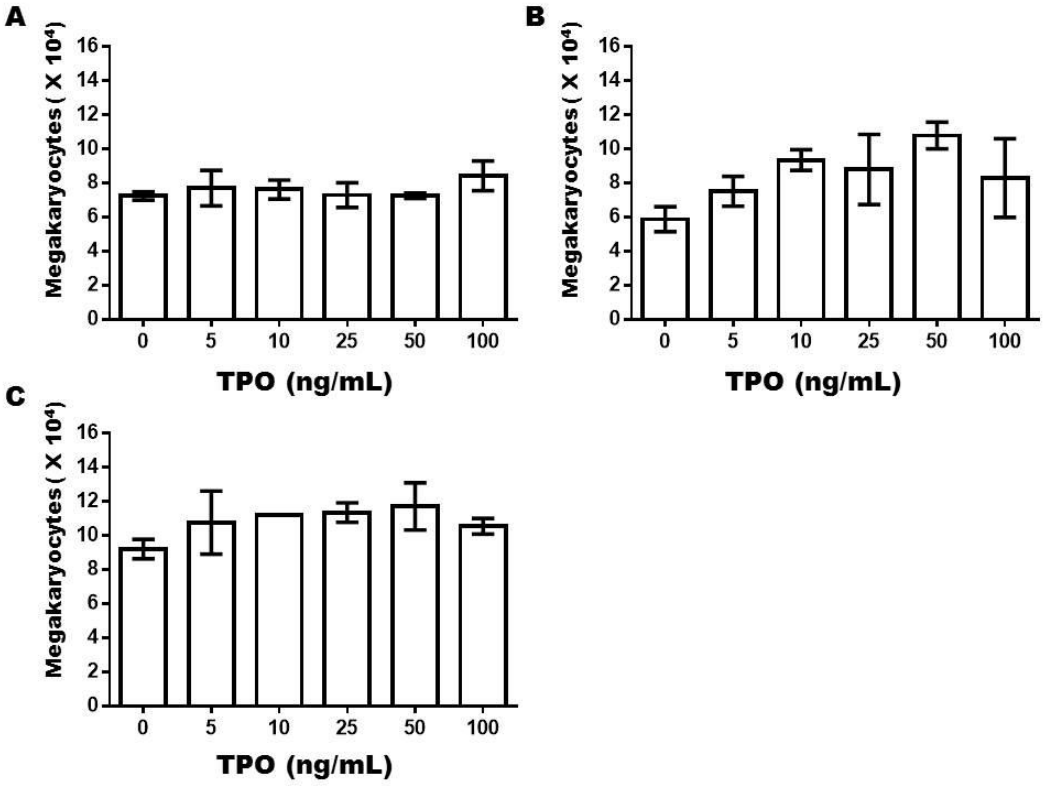


Figure 6. Investigation of megakaryocyte growth response to TPO doses in megakaryocyte cultures set up with *ex vivo* expanded CD34+ cells: megakaryocyte cultures were set up with *ex vivo* expanded CD34+ cells from peripheral blood in presence of increasing TPO concentrations (0, 5, 10, 25 and 100 ng/mL). Megakaryocytes were enumerated by multiplying total number of live cells (measured by trypan blue exclusion) by the percentage of flow cytometry CD41/61+ events at day (A) 8 (B) 11 and (C) 13. No significant difference in number of megakaryocytes was found at any day of analysis. Experiments were set up in duplicate from one donor. ANOVA analysis was used to compare the megakaryocyte growths on each day.

3.4 Megakaryocyte cultures with micro volume cultures:

A megakaryocyte culture system was developed using unexpanded PB-CD34+ cells in 96-well tissue-culture plates. It was expected that this method using smaller volumes would be more efficient compared to megakaryocyte cultures in 24-well tissue-culture plates while maintaining the megakaryopoiesis response to TPO doses.

3.4.1 Optimization of seeding number of CD34+ cells:

In order to determine the minimum number of seeding CD34+ cells, cultures with seeding numbers of 2×10^3, 5×10^3,$1x10^4$ and 2×10^4 CD34+ cells/well were set up in the presence of TPO (50 ng/mL) in duplicate. Cultured cells were enumerated on day 8 and 11 by trypan blue exclusion. Only wells with the seeding number of 1×10^4 and 2×10^4 CD34+ cells/well had a detectable number of cells with viability over 80%. Therefore, 1×10^4 CD34+ cells/well (in 200 µL) were used as the seeding number of CD34+ cells in future experiments with 96-well tissue-culture plates.

3.4.2 Megakaryocyte production in micro volume experiments:

The presence of large and proplatelet forming cells on day 12 of cultures set up in the presence of TPO (50 ng/mL) were suggestive of megakaryocyte production (figure 7). Bright field microscopic examination of H and E stained

cells from the same culture showed the presence of cells with the same morphological features as megakaryocytes (large polyploid cells) (figure 8).

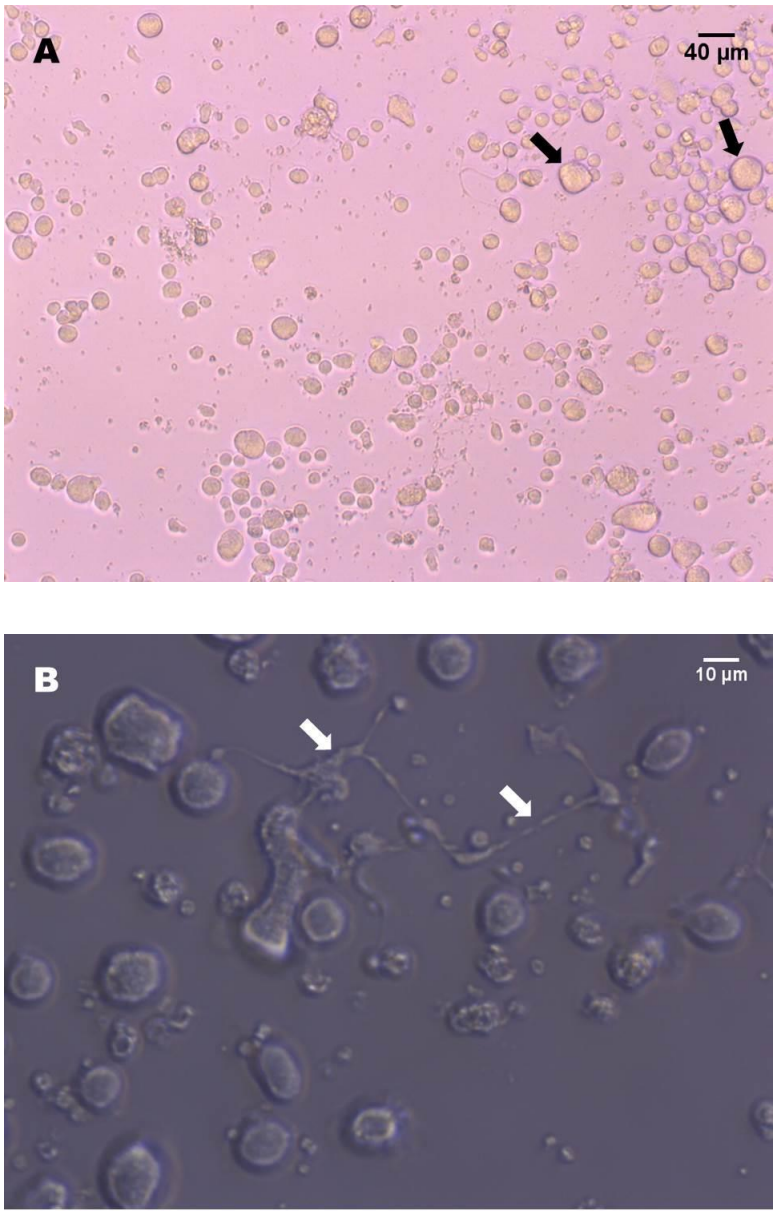


Figure 7. Megakaryocyte production in micro volume megakaryocyte cultures: Live microscopic images of a culture at day 12 set up with PB-CD34+ cells (purity: 37%) in the presence of TPO (50 ng/mL) in 96-well tissue-culture plates. Arrows show the presence of (A) large cells and (B) proplatelet forming cells which were suggestive of megakaryocyte production in this culture.

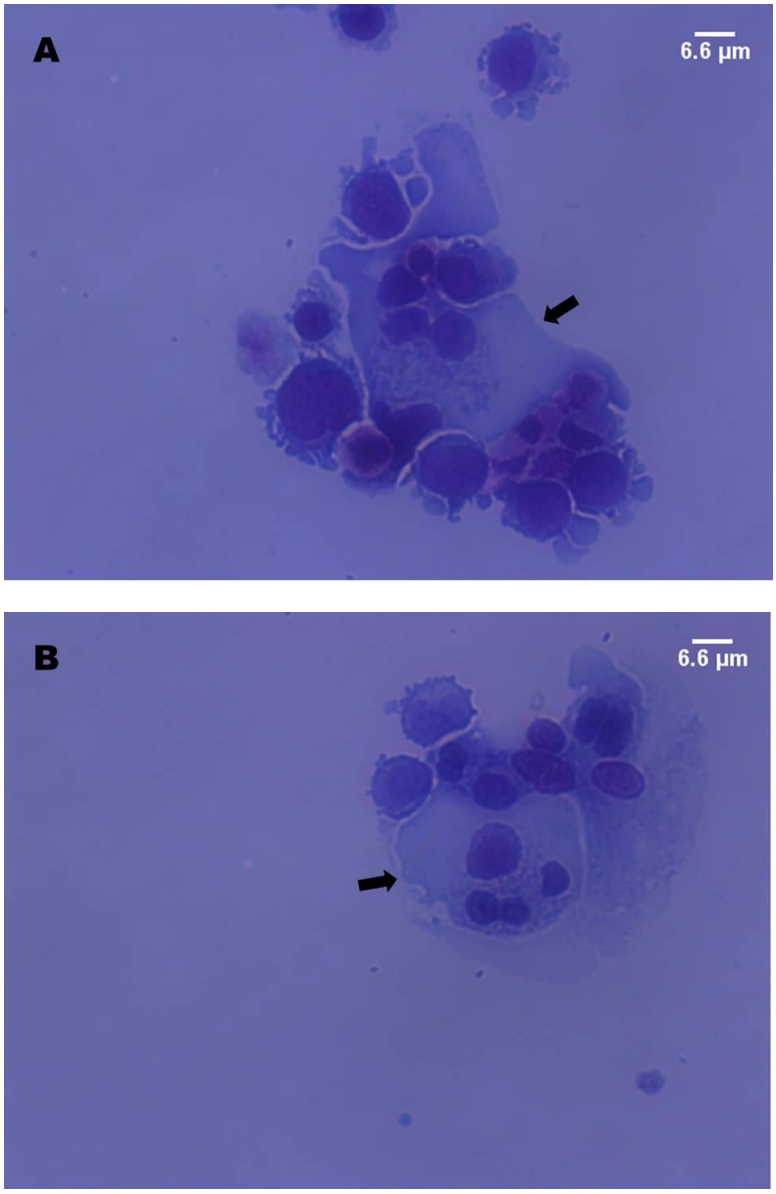


Figure 8. Production of large polyploid cells in micro volume megakaryocyte cultures: Bright field microscopic images of H and E stained cells in a culture (same culture as figure 7) at day 12 shows production of cells with morphological features of megakaryocytes (large polyploid cells). Arrows in image A and B point to large polyploid cells.

Flow cytometry analysis showed that 94.5% of cultured cells differentiated to megakaryocytes (CD41/61+ cells) and 81.0% were mature megakaryocytes (CD41/61+CD42+ cells) (figure 9).

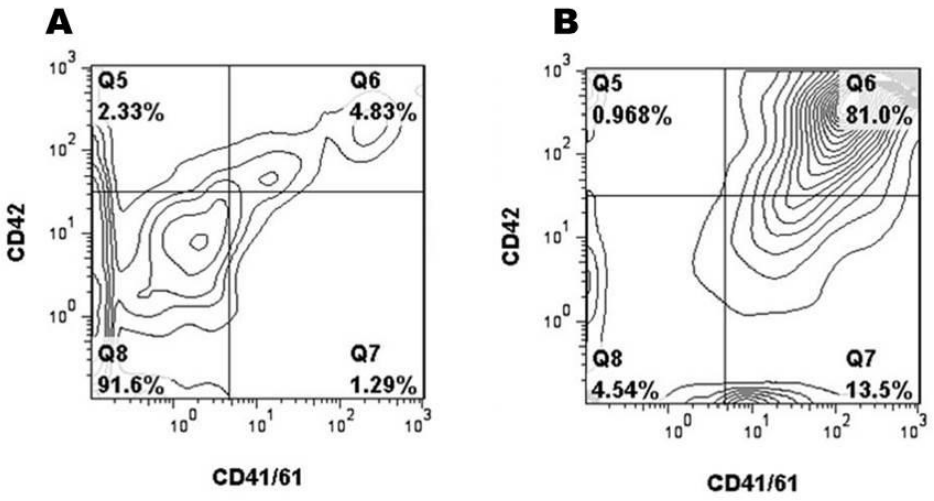


Figure 9. Expression of megakaryocyte markers in micro volume megakaryocyte cultures: Flow cytometry analysis of cells in the culture at day 12 showed expression of CD41/61 and CD42 markers. Dot-plot graphs of A) day 0 and B) day12 cultures showing the expression of CD41/61 and CD42 markers. 2000 events were acquired for flow cytometry analysis.

No significant difference existed between the average purity of CD34+ cells which produced megakaryocytes in micro volume megakaryocyte cultures (54.9%, n = 8) and the average purity with no growth (37.8%, n=4) (figure 10).

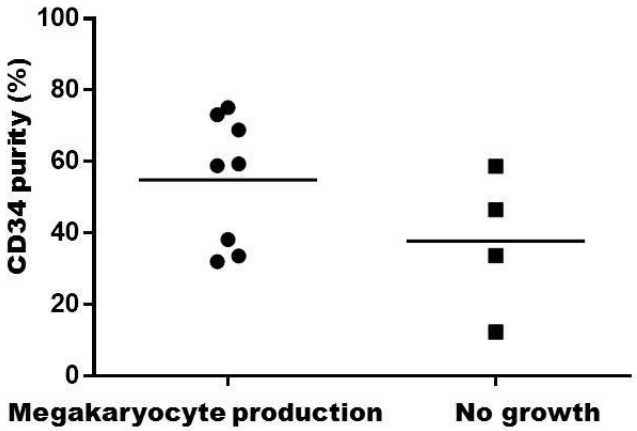
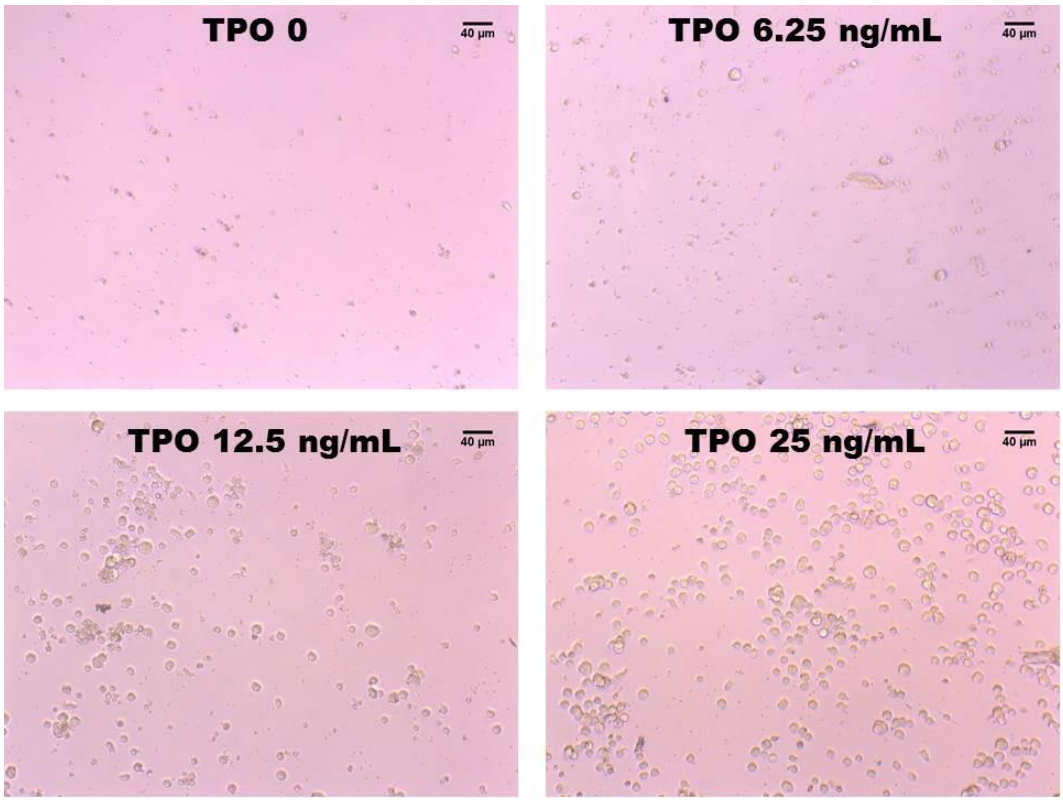


Figure 10. The comparison of CD34+ cell purity between PB-CD34+ samples with megakaryocyte production and samples without any growth in micro volume cultures: Among 12 cultures set up with PB-CD34+ cells in 96-well tissue-culture plates, a group of cultures (8/12) produced megakaryocytes with the average PB-CD34+ cell purity of 54.9% while some cultures (4/12) had no cell growth (average PB-CD34+ cell purity of 37.8%). No significant difference existed between the purity of the two groups. Bars show the mean of CD34+ cell purity in each group. Each point represents one culture set up in triplicate in the presence of TPO (20 ng/mL). Megakaryocytes were detected by the presence of large cells at day 11 or 12 by live microscopic examination of cultures and confirmed by flow cytometry and/or immunofluorescence confocal microscopy.

3.4.3 Megakaryopoiesis response to TPO doses in micro volume megakaryocyte cultures:

In order to investigate the megakaryopoiesis-TPO dose response, megakaryocyte cultures were set up in the presence of increasing TPO concentrations (0, 6.25, 12.5, 25 and 50 ng/mL) in triplicate for 12 days in 96-well tissue-culture plates (n = 3, three different donors). Live microscopic examination of cultures showed an increase in number and size of cells at day 12 that correlated with increasing TPO concentration in cultures (figure 11). There was no cell growth in the absence of TPO. CD41/61 expression in cultured cells also increased as TPO concentration increased (figure 12).



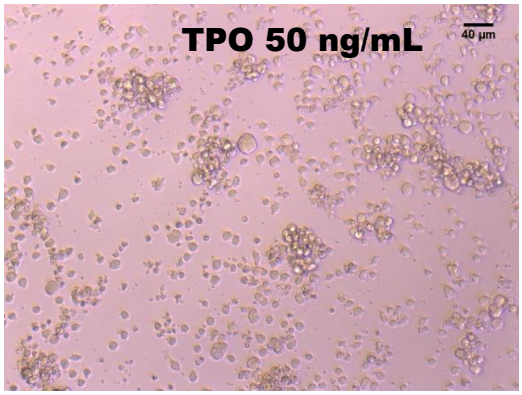


Figure 11. Live microscopic examination of cells for investigation of cell growth response to increasing TPO concentration in micro volume megakaryocyte cultures: Live microscopic images of cells at day 12 in 96-well tissue-culture plates showed an increase in number and size with increasing TPO concentration. Cultures set up in the absence of TPO showed no growth. Cultures were set up with PB-CD34+ cell purity of 68.9%.

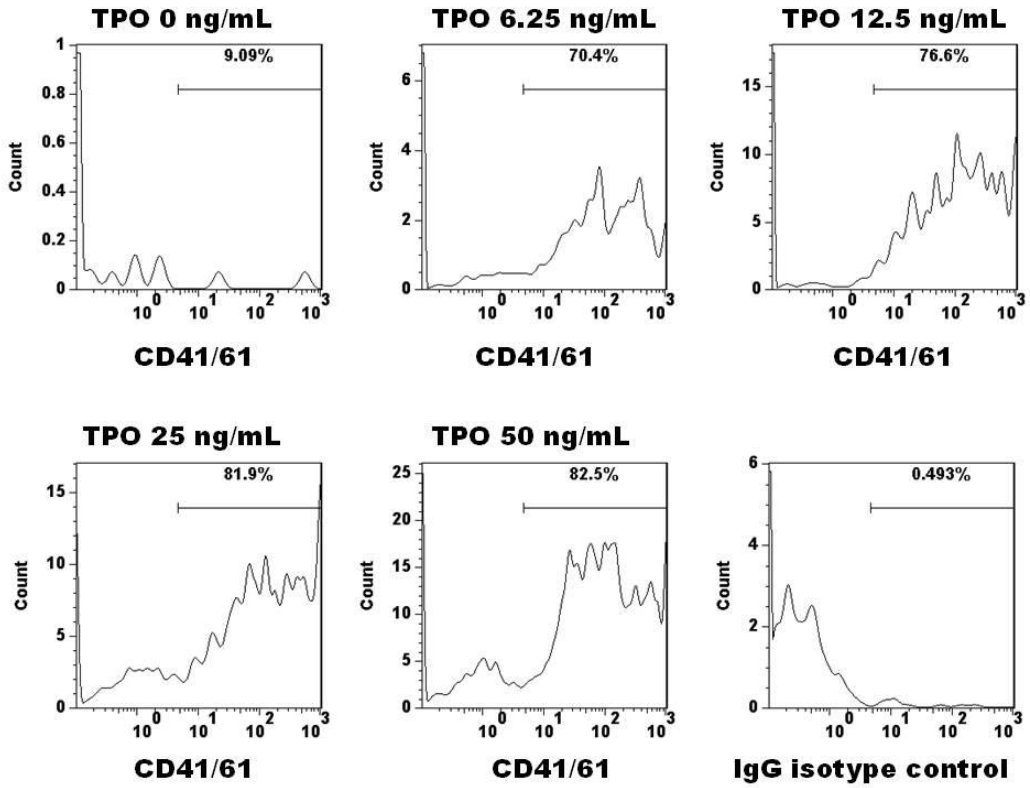


Figure 12. Expression of CD41/61 marker in response to increasing TPO concentration in micro volume megakaryocyte cultures: flow cytometry analysis showed an increase in CD41/61 marker consistent with increasing TPO concentration. Cultures were set up with PB-CD34+ cell purity of 68.9%. 1500-2500 flow cytometry events were acquired with the exception of cultures grown with TP 0 and 6.25 ng/mL for which only 100-600 events could be acquired.

In order to find the optimal TPO concentration in micro volume megakaryocyte cultures, megakaryopoiesis response to various TPO concentrations was investigated (n = 3, different donors). Significant differences were found in the number of megakaryocytes between cultures grown with TPO 0 and 6.25 ng/mL (p-value = 0.0178) and 12.5 and 25 ng/mL (p-value = 0.0379) (figure 13 A). The number of mature cells (CD41/61+CD42+) cells was also significantly different between cultures grown with TPO 0 and 6.25 ng/mL (p-value = 0.0305), 6.25 ng/mL and 12.5 ng/mL (p-value = 0.0121) and 12.5 and 25 ng/mL (p-value = 0.0207) (figure 13 B). In conclusion, the production of megakaryocytes is TPO dependent in micro volume cultures.

An optimal TPO dose was defined as a concentration of TPO which induced the production of highest number of megakaryocytes without saturation in megakaryopoiesis. Because TPO 12.5 and 25 ng/mL were significantly different in megakaryopoiesis response while 25 and 50 ng/mL were not, the optimal TPO dose lies in between TPO 12.5 and 25 ng/mL. Therefore, for future experiments 20ng/mL was used as the optimal TPO concentration.

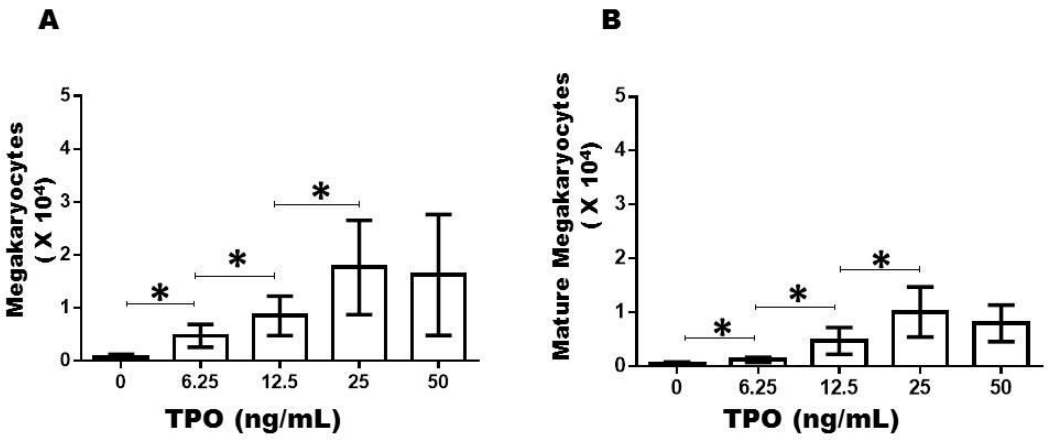


Figure 13. Investigation of megakaryopoiesis-TPO dose response in micro volume megakaryocyte cultures: Total number of megakaryocytes and mature megakaryocytes were compared between each TPO concentration. (A) Total number of megakaryocytes were significantly different between consecutive TPO concentrations of TPO 0 and 6.25ng/mL (p-value = 0.0178) and TPO 12.5 and 25ng/mL (p-value = 0.0379) as shown on the graph. Also there is a significant difference between TPO 0 and 25 ng/mL (p-value = 0.0002) and TPO 0 and 50 ng/mL (p-value = 0.0002) (B) Total number of mature megakaryocytes were significantly different between consecutive TPO concentration of TPO 0 and 6.25ng/mL (p-value = 0.0305), TPO 6.25and 12.5ng/mL (p- value = 0.0121), TPO 12.5 and 25ng/mL (p-value = 0.0207) as shown on the graph. Also there is a significant difference between TPO 0 and 25 ng/mL (p-value = 0.0002) and TPO 0 and 50 ng/mL (p-value = 0.0002). Results are the pool of experiments from three donors (CD34+ cell purities: 68.9, 37 and 50%) set up in triplicate. Cultured cells were enumerated by multiplying total number of live cells (measured by trypan blue exclusion) by percentage of CD41/61+ cells (for megakaryocyte enumeration) or CD41/61+CD42+ cells (mature megakaryocytes) in flow cytometry analysis. Error bars represent SD. P-values were calculated using Mann-Whitney test. The asterisk, *, represents p-value ≤ 0.05

3.4.4 Optimal end point of micro volume megakaryocyte cultures:

In order to determine the optimal timeline, megakaryocyte cultures were set up with TPO (20 ng/mL) in triplicate in 96-well tissue-culture plates and cells were analyzed on day 3, 5, 8, 11, 12, 13 and 16 (n = 2, different donors). The average viability of cells from day 0 to day 13 was 92.5 ± 2.4%, while the average viability significantly decreased to 62.8 ± 5.1 on day 16 (p-value = 0.0014)(figure 14).

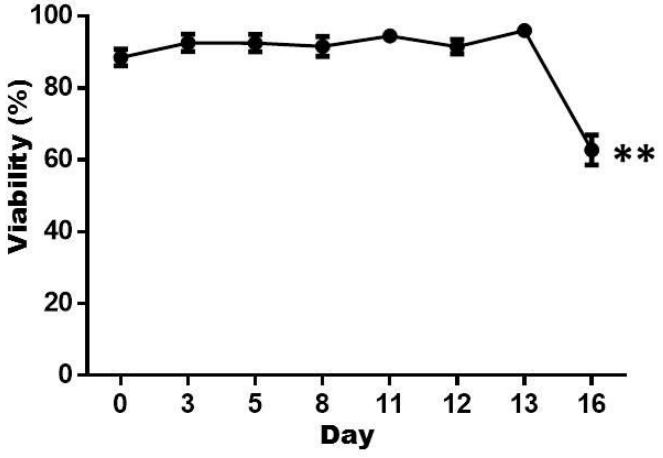


Figure 14. The viability of cells during the course of micro volume megakaryocyte cultures: The average viability at day 16 was significantly different than the viability at day 0 to 13 (p-value = 0.0014). Results are the pool of experiments from two donors set up in triplicates with PB-CD34+ cell purities of 68.9 and 73.1%. Cultures were grown in presence of TPO (20 ng/mL) in 96-well tissue-culture plates. The viability was measured using trypan blue exclusion.

Error bars represent SD. P-value was calculated using ANOVA test. Double asterisks, **, represent p-value ≤ 0.01.

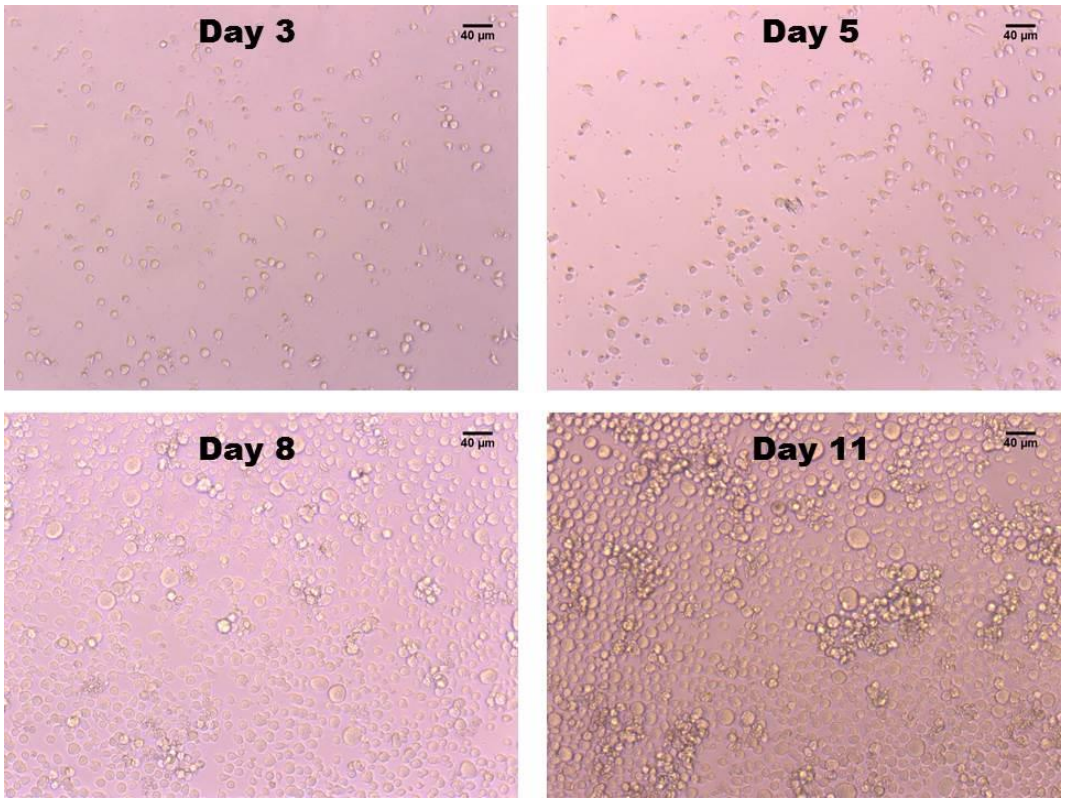
Live microscopic examination of cultures showed that at day 3 and 5, cells were relatively small and in lower numbers compared to day 8-13. Large cells suggestive of the presence of megakaryocytes were present at day 8-13. Day 16 Cultures contained clumps of fragmented cells which is a characteristic of low viability in culture (figure 15).

Flow cytometry analysis also illustrated an increase in both size and granularity from day 0 to day 8; however, day 11 to day 13 analysis showed similar size and granularity (figure 16).

The expression of progenitor and megakaryocytic markers showed a gradual decrease in CD34 (the hematopoietic progenitor marker) from day 5 to day 13 while megakaryocytic marker (CD41/61+ and maturation marker (CD41/61+CD42+) gradually increased from day 0 and peaked at day 11 reaching 90.65% and 76.10%, respectively (figure 17). Comparison of CD41/61 expressions throughout the course of culture, showed significant differences between day 3 and 5 (p-value = 0.0022), day 5 and 8 (p-value = 0.0022), and day 8 and 11 (p-value = 0.0043). No significant difference was found in CD41/61 expression between day 11 and 12 and day 12 and 13 (n = 2). Also, Comparison of CD41/61+CD42+ expression showed significant differences between day 3

and 5 (p-value = 0.0022), day 5 and 8 (p-value = 0.0022), and day 8 and 11 (p-value = 0.0022).

Images from immunofluorescence confocal microscopy illustrated surface expression of CD41/61 and CD42 confirming the flow cytometry results (figure 18).



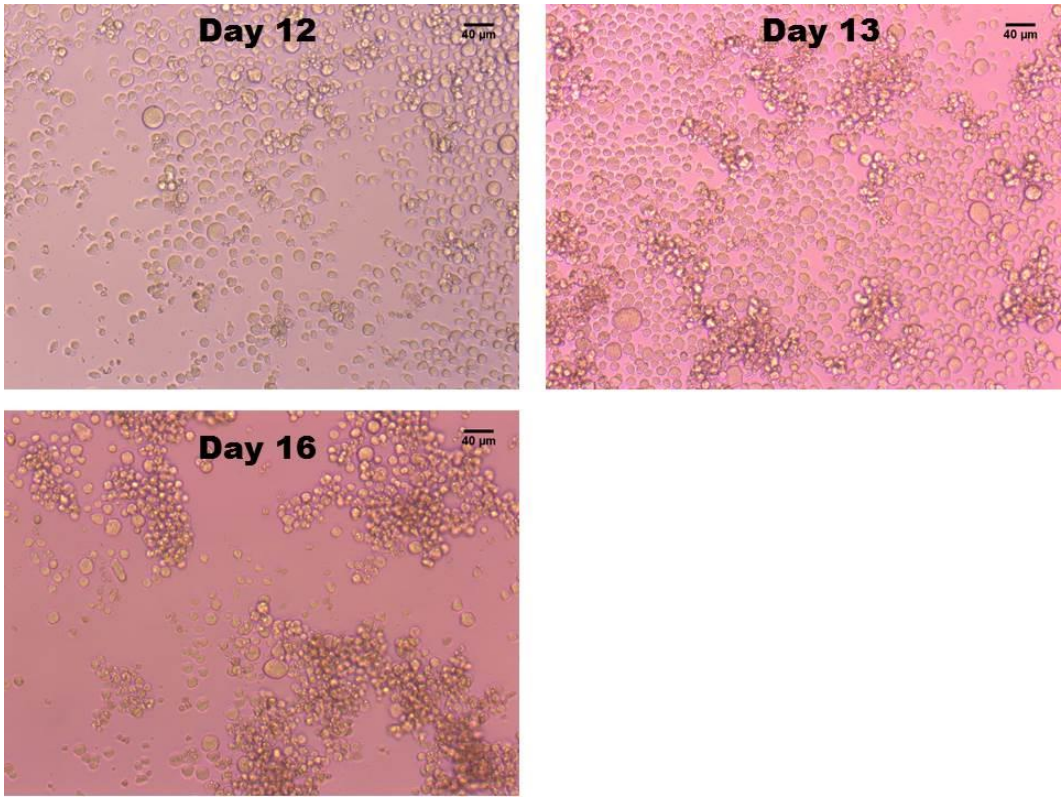


Figure 15. Live microscopic examination of cells throughout the course of micro volume megakaryocyte cultures: Live microscopic images showed changes in number and size of cells throughout the course of culture. From day 3 to 5, there was an increase in size and number of cells whereas images of cells from day 8 to day 13 showed little difference. The culture on day 16 had clumps and fragmented cells suggesting low viability at this day. Cultures were set up with PB-CD34+ cells (purity: 68.9 %) in presence of TPO (20 ng/mL) in 96-well tissue-culture plates.

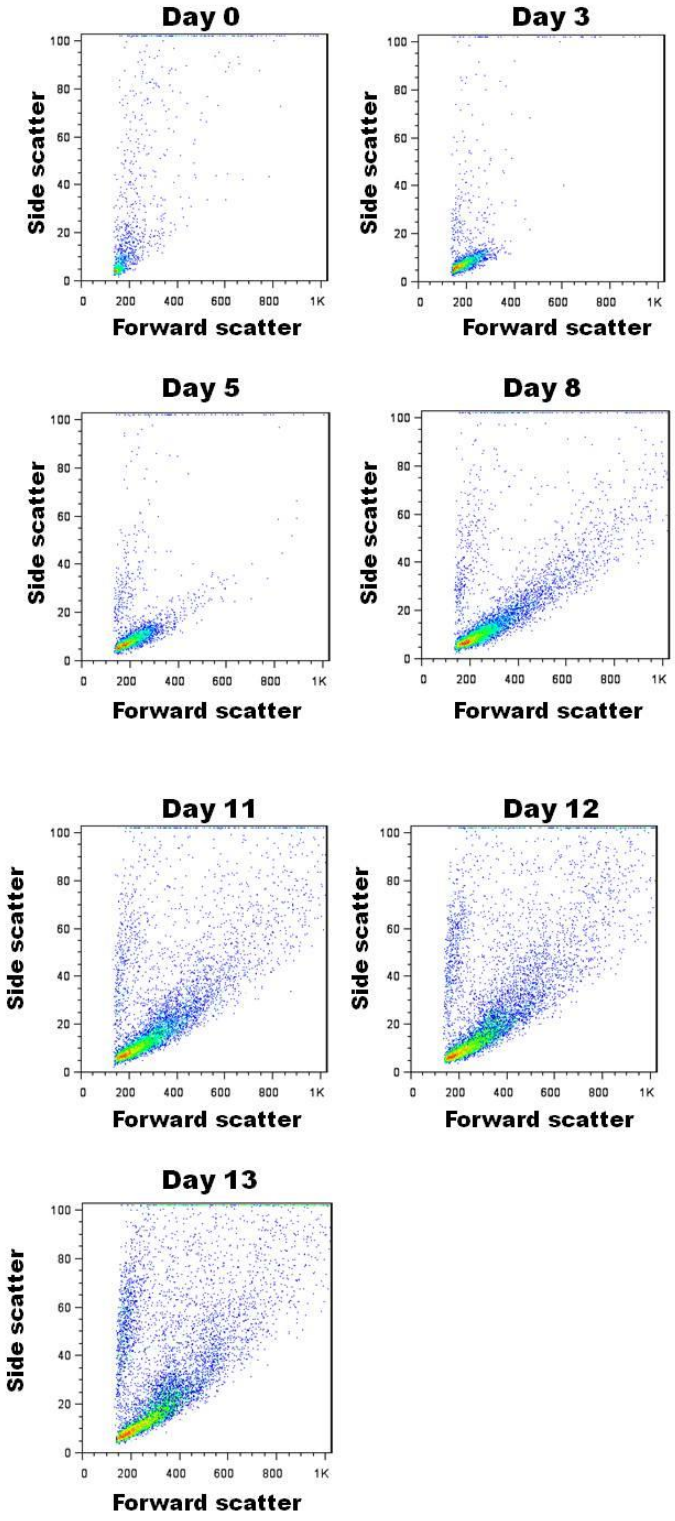

Day 0
Day 3
Day 5
Day 8
Day 11
Day 12
Day 13
Side scatter
Forward scatter
0
20
40
60
80
100
200
400
600
800
1K

Figure 16. Size and granularity analysis throughout the course of micro volume megakaryocyte cultures: Flow cytometry analysis showed an increase in both size and granularity from day 0 to day 8 while cultures from day 11 to 13 had similar size and granularity. Forward and side scatter represent size and granularity, respectively. Cultures were set up in triplicate with PB CD34+ cells (purity: 68.9 %) in presence of TPO (20 ng/mL) in 96-well tissue-culture plates. 10000 events were acquired for flow cytometry analysis except for day 0-5 for which only 2000-5000 events could be acquired.

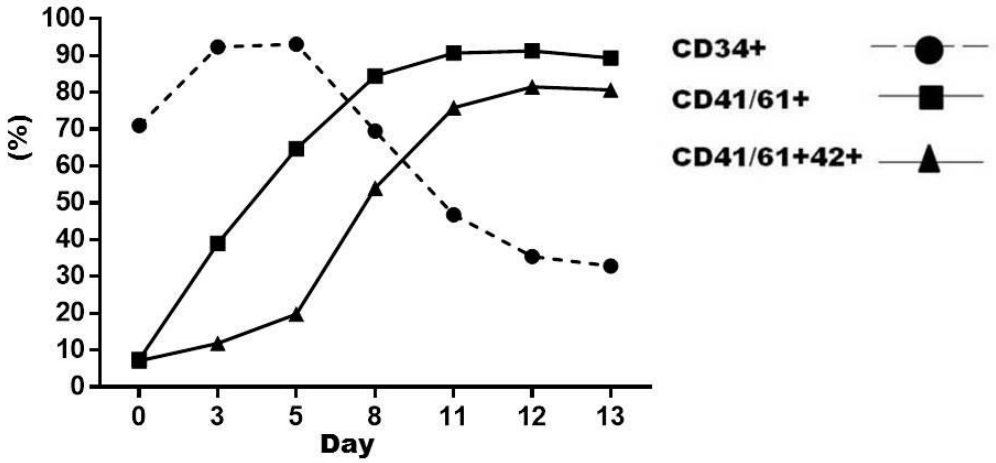


Figure 17. The expression of hematopoietic progenitor and megakaryocytic markers throughout the course of micro volume megakaryocyte cultures: Flow cytometry analysis showed the changes in expression of markers from day 0 to 13. Hematopoietic progenitor marker (CD34) gradually decreased from day 5 to day 13 while megakaryocyte lineage marker (CD41/61) and maturation marker (CD41/61+CD42+) increased and peaked on day 11. Results are the pool of experiments set up in triplicate. Cultures were set up in the presence of TPO (20 ng/mL) with PB-CD34+ cells from two donors (purities: 68.9 and 73.1%). The percentage of each marker was obtained from flow cytometry analysis. 10000 events were obtained for each analysis except for day 0-5 for which only 2000-5000 events could be acquired.

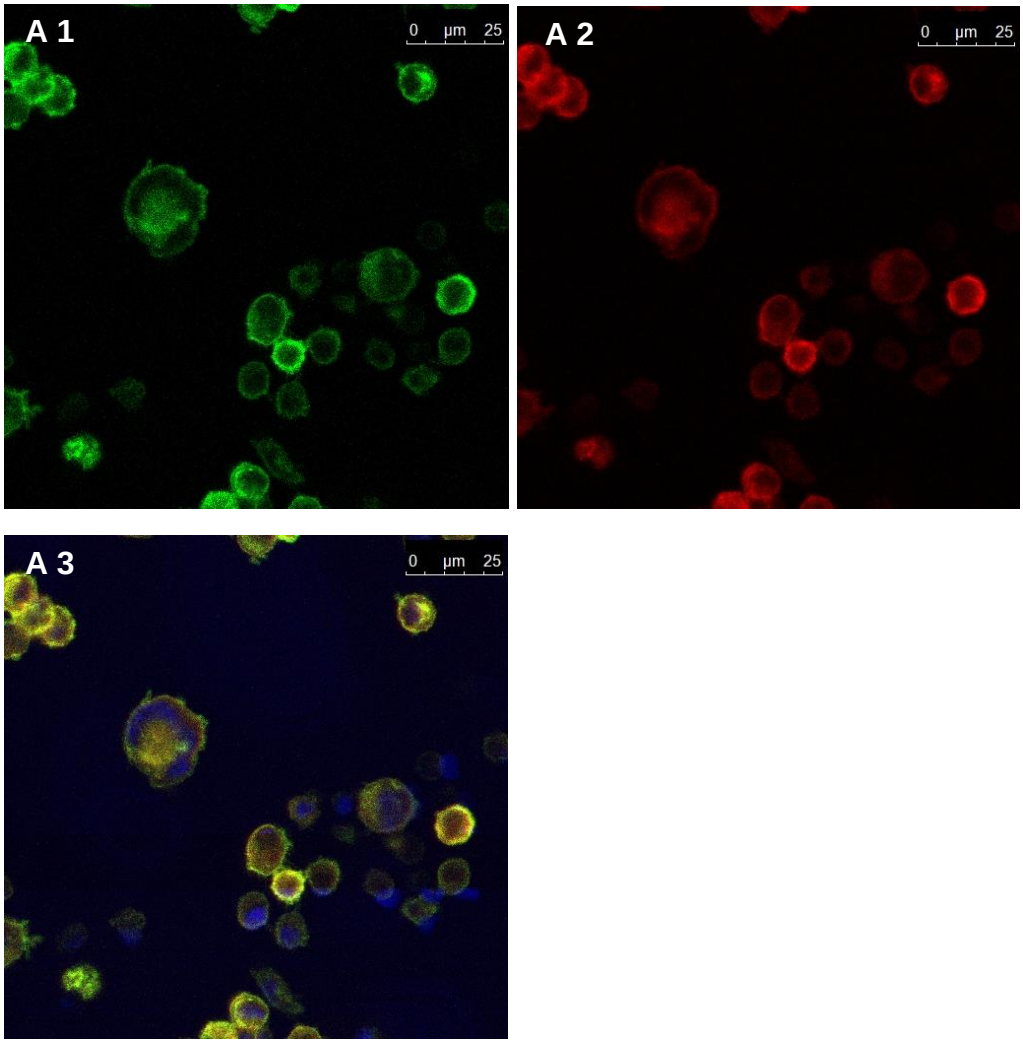


Figure 18. Immunofluorescence confocal microscopy of cells at day 11 in micro volume megakaryocyte cultures: Confocal microscopy images of immunofluorescence stained cells at day 11 of culture showed surface expression of CD41/61 (A1, green) and CD42 (A2, red). A3 image shows the overlay image of A1 and A2 with the background staining of nucleus (blue). The culture was set up in the presence of TPO (20 ng/mL) with PB-CD34+ cells with purity of 73.1%. Cells were fixed with PFA. Cells were stained by direct staining with FITC conjugated mouse anti-human CD41/61(detected by green colour) and indirect staining with primary mouse anti-human CD42 and TRITC conjugated goat anti-mouse (detected by red colour). DAPI was used for background and nucleus staining (detected by blue colour).

Analysis of the total number of megakaryocytes and mature megakaryocytes demonstrated that the production of CD41/61+ cells was significantly different between day 3 and 5 (p-value = 0.0260), day 5 and 8 (p-value = 0.0022) and day 8 and 11 (p-value = 0.0043) while there was no difference between day 11 and 12 and day 12 and 13 (figure 19-A). Comparison of total number of CD41/61+CD42+ cells (mature megakaryocytes) showed significant differences between day 3 and 5 (p-value = 0.0022), day 5 and 8 (p-value = 0.0022), and day 8 and 11 (p-value = 0.0022) as illustrated by figure 19-B.

Polyploidy distribution was investigated at day 8 to 13 by measuring total DNA content (figure 20). Total level of polyploidy (DNA content ≥ 4N) was highest at day 8 and 11 (average total polyploidy: 50.1% and 38.6%, respectively) compared to day 12 and 13 (average total polyploidy: 31.2 and 26.7%, respectively). The highest percentage of cells with 16N was observed at day 8 and 11 (4.3% and 4.2%, respectively) compared to day 12 and 13 (1.6 and 2.0% respectively) (n = 2, different donors).

Even though the polyploidy levels are highest on day 8, but the number of CD41/61+ and CD41/61+CD42+ cells are significantly higher on day 11 compared to day 11. Additionally, the expression of CD41/61+ and CD41/61+CD42+ markers reached the maximum on day 11, therefore day 11 was chosen as the optimal day for the micro volume megakaryocyte culture.

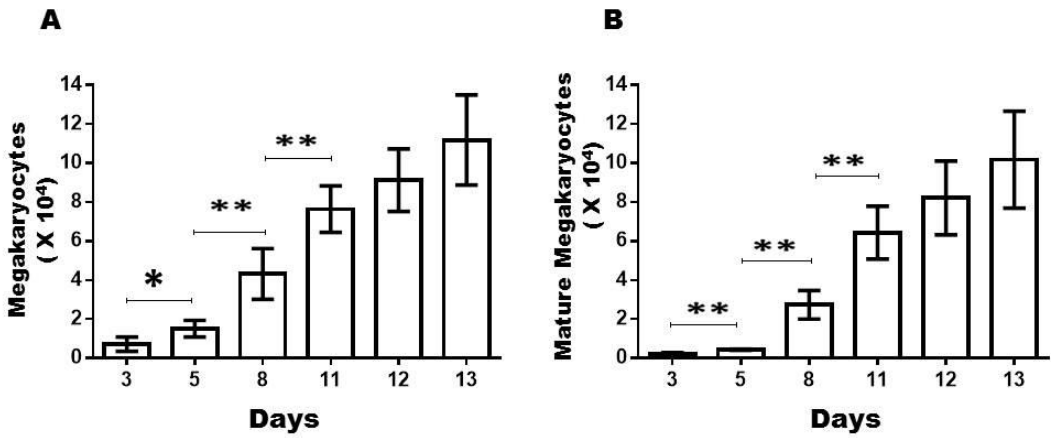
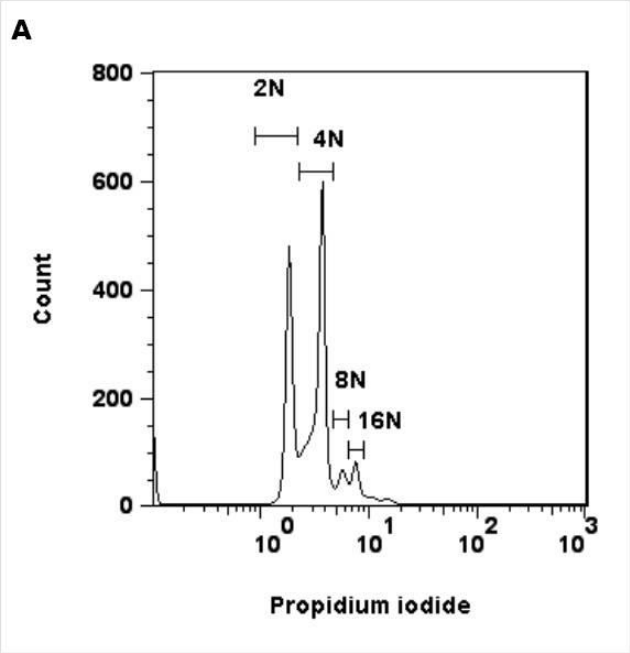


Figure 19. Investigation of megakaryopoiesis and days of culture in order to determine the optimal endpoint for micro volume megakaryocyte cultures: (A) Comparison of total number of megakaryocytes showed significant differences between day 3 and 5 (p-value = 0.0260), day 5 and 8 (p-value = 0.0022), and day 8 and 11 (p- value = 0.0043) as illustrated on the graph. (B) Comparison of total number of CD41/61+CD42+ cells showed significant differences between day 3 and 5 (p-value = 0.0022), day 5 and 8 (p-value = 0.0022), and day 8 and 11 (p-value = 0.0022) as illustrated on the graph. Cells were enumerated by multiplying total number of live cells (measured by trypan blue exclusion) by percentage of CD41/61+ cells (megakaryocytes) or CD41/61+CD42+ cells (mature megakaryocytes) in flow cytometry analysis. Cultures were set up in triplicates from two donors (PB-CD34+ cell purities: 68.9 and 73.1%) in the presence of TPO (20 ng/mL) in 96-well tissue-culture plates. Error bars represent SD. P-values were calculated using Mann-Whitney test. The asterisk, *, represents p-value ≤ 0.05 and double asterisks, **, represent p-value ≤ 0.01.


A
800
600
400
200
0
Count
2N
4N
8N
16N
10^0
10^1
10^2
10^3
Propidium iodide

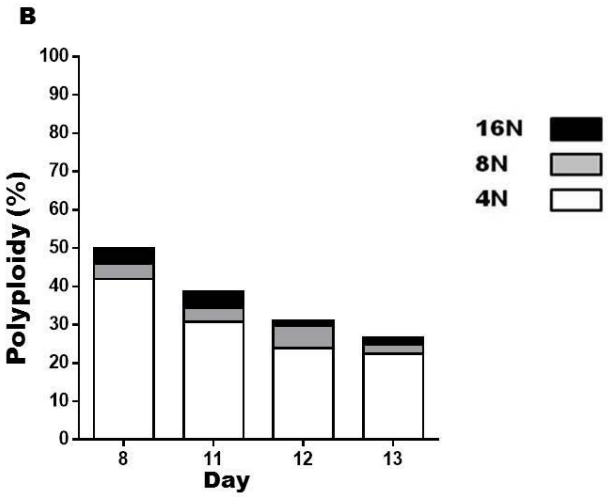

B
100
90
80
70
60
50
40
30
20
10
0
Polyploidy (%)
16N
8N
4N
8
11
12
13
Day

Figure 20. Polyploidy distribution: (A) the flow cytometry histogram showing the binding of PI in cultured cells at day 8 as a measure of polyploidy (DNA content). Each peak represents the percentage of cells with the associated ploidy (2N, 4N, 8N or 16N). (B) Total polyploidy (ploidy ≥4N) and the distribution of ploidy at each day of culture (day 8-13). Columns represent the result of two flow cytometry analysis from two donors. 3000-5000 flow cytometry events were acquired for each analysis. Cultures were set up from two donors (PB-CD34+ cell purities: 68.9 and 73.1%) in presence of TPO (20 ng/mL) in 96 well tissue-culture plates.

CHAPTER 4: DISCUSSION

4.1 Summary of the development of a human megakaryocyte culture suitable for platelet disorder studies:

The efforts in developing a megakaryocyte culture from human PB-HPCs suitable for ITP studies and large scale experiments have been summarized in table 5.

Type of Culture	Megakaryocyte production	High efficiency	TPO-dependent	Alternative method
PB-CD34+ Cells in 24-well tissue-culture plates	Yes	No	Yes	Develop a method to produce higher number of CD34+ cells
ex vivo expanded PB-CD34+ Cells in 24-well tissue-culture plates	Yes	Yes	No	Expansion of CD34+ cells diminishes the TPO-dose response. Develop a method to utilize unexpanded PB-CD34+ cells in a high efficiency manner.
PB-CD34+ Cells in 96-well tissue-culture plates	Yes	Yes	Yes	

Table 5: The Summary of the development of a megakaryocyte culture from peripheral blood HPCs suitable for studies of ITP and large scale experiments

By applying 96-well tissue-culture plates, we were able to develop a method to produce megakaryocytes *in vitro* with high purity utilizing human PB-HPCs in a high efficiency manner. The production of cells with similar morphology (large polyploid cells) to megakaryocytes (as found in bone marrow slides) and formation of proplatelet structures (similar to proplatelets identified by other investigators) confirms the production of mature and functional megakaryocytes *in vitro* with this culturing system (figure 7 and 8) (American Society of Hematology, 2013, Italiano et al., 1999).

The megakaryocyte growth and maturation were significantly different between TPO concentrations of 0-25 ng/mL confirming the presence of a megakaryopoiesis-TPO dose response within this range (figure 13). The TPO concentration of 20 ng/mL was determined as the optimal TPO concentration which supports the production of mature megakaryocytes with high megakaryocytic purity without reaching saturation in megakaryopoiesis response to TPO concentrations.

In a study by Yasui et al., even though they used SCF and a different TPO concentration, the kinetic of CD41/61 expression and polyploidy levels were similar to our results: CD41/61 expression increased from day 0-10 and plateaued after day 10 (day 11 in our results, figure 18) and polyploidy increased up to day 10 to a level of 16N (day 11 in our results, figure 21) and decreased in the following days (Yasui et al., 2003). In our experiments, the expression of CD41/61+ and CD41/61+CD42+ markers reached the maximum on day 11, while the polyploidy levels at this day was higher than day 12 and 13, therefore day 11 was taken as the optimal endpoint for this megakaryocyte culture for the study of megakaryocyte growth and maturation *in vitro*.

4.2 The micro volume megakaryocyte culture is a novel assay for investigating and identifying ITP plasma/antibodies with suppressive effects on normal megakaryopoiesis:

Impairment of platelet production by autoantibodies has been considered as one of the pathogenic mechanisms in ITP (Arnold et al., 2009). Several studies applied megakaryocyte cultures to investigate the suppressive effects of ITP plasma/antibodies on normal megakaryopoiesis; however, due to differences in culture conditions and lack of optimization, the results were divergent (Chang et al., 2003, McMillan et al., 2004, Yang et al., 2010). The megakaryocyte cultures used by McMillan et al. and Chang et al. showed a megakaryopoiesis-TPO dose response. However, due to using sub-optimal TPO concentrations, the megakaryocyte yield and maturation was low in these studies and therefore it was not possible to investigate the suppressive effects of ITP plasma/antibodies on all stages of megakaryopoiesis (Chang et al., 2003, McMillan et al., 2004). Here, we found a TPO concentration and culture conditions which supports all the stages of megakaryocyte growth and maturation with a megakaryocytic yield over 90% allowing the investigation of ITP sera and other inhibitory factors effects on all stages of megakaryopoiesis.

Yang et al. was able to investigate the megakaryocyte growth and maturation as well as *in vitro* platelet production. However, due to the use of a high TPO concentration in their culture, the suppression of megakaryopoiesis by ITP plasma/antibodies may have been masked (Yang et al., 2010). More

importantly, the presence of a high concentration of SCF, which is known to have restoring effects on megakaryocytes in the presence of inhibitory agents may have further boosted megakaryopoiesis and as the result, may have diminished the ability of this culture system to detect the suppression in megakaryopoiesis (Zeuner et al., 2007). In our megakaryocyte culture system, we found a TPO concentration which supports the maturation of megakaryocytes while the megakaryopoiesis response is TPO dependent and therefore allows the investigation of suppressive effects of ITP plasma/antibodies on normal megakaryopoiesis.

Even though the optimal TPO concentration of 20 ng/mL doesn't reach saturation in megakaryopoiesis response, the use of this TPO concentration must be evaluated by a known plasma/antibody with known suppressive effects to ensure this TPO concentration doesn't mask the effects of ITP antibodies/plasma.

4.3 The use of mobilized or *ex vivo* expanded CD34+ cells in megakaryocyte cultures must be carefully evaluated for investigation of megakaryopoietic inhibitors or studies requiring TPO sensitivity:

In this study, no megakaryopoiesis-TPO dose response was found in megakaryocytes produced from *ex vivo* expanded CD34+ cells suggesting the expansion of CD34+ cells with cytokines such as SCF and GM-CSF reduces the TPO dependency in megakaryocytes (figure 6). In mobilization of peripheral

blood, G-SCF is used to induce the mobilization of CD34+ cells from bone marrow and increase the frequency of CD34+ cells in peripheral blood (Motabi et al., 2012). As previously mentioned, mobilized blood is a rich source of CD34+ cells and these cells are used for *in vitro* production of megakaryocytes; however, since the TPO-dependency of megakaryopoiesis may be reduced due to exposure of CD34+ cells to mobilizing cytokines, it is critical to carefully evaluate the megakaryopoiesis-TPO dose response in studies using mobilized CD34+ cells in megakaryocyte cultures for investigation of megakaryopoiesis in ITP, new megakaryopoietic agents or inhibitory factors.

4.4 The utilization of PB-CD34+ cells in a high efficiency manner:

CD34+ cells are important components of megakaryocyte cultures which can affect the outcome of cultures. With the exception of bone marrow, CD34+ cells can be obtained from blood samples with moderately invasive (PB or mobilized blood) or non-invasive (cord blood) collection methods; however, the accessibility to a source of CD34+ cells can be limited and require affiliation or collaboration with clinical personnel and facilities (Rodak et al., 2007, Mehrishi and Bakács, 2013). Peripheral blood is the most accessible source of CD34+ cells which can also be obtained outside of clinical settings and is available to all researchers and research facilities. CD34+ cells also have several advantages in megakaryocyte cultures: 1) they have a higher percentage of myeloid progenitors

compared to bone marrow (Fritsch et al., 1995) 2) they have a higher potential for megakaryocytic differentiation and maturation comparing to cord blood (Cho et al., 1999, Mattia et al., 2009) 3) they require less time in culture to produce mature megakaryocytes (Schipper et al., 2003). The major limitation associated with the use of peripheral blood as the source of CD34+ cells is the significantly low frequency of CD34+ cells in peripheral blood compared to other sources which result in low yield and purity (Cohen et al., 2013). In this study, 180mL of whole blood had yielded the average of 5.5×10^5 CD34+ cells (n = 18) and by applying 96-well tissue-culture plates and using 1×10^4 CD34+ cells/well (200 µL) , the average of 13 experiments in triplicate can be set up.

We were also able to demonstrate the production of megakaryocytes from PB-CD34+ cells with purities between 32.0%-75.1% (n = 8) (figure 10) which supports the conclusion that a CD34 purity ≥ 90% is not a necessity for the production megakaryocytes from PB-CD34+ cells.

4.5 The limitations associated with the micro volume megakaryocyte culture:

A great variation was observed in the purity and the yield of isolated CD34+ cells. Since the yield of isolated CD34+ cells depends on the available number of CD34+ cells in the peripheral blood, the variations can be explained by the significant variation of CD34+ cell frequency in donors (Cohen et al., 2013). A

combination of genetic and environmental factors as well as age and gender affect the frequency and number of CD34+ cells in peripheral blood (Cohen et al., 2013). Even though the average purity of CD34+ cells with megakaryocyte production (54.9%, n = 8) was higher than the purity of CD34+ cells without any growth (37.8 %, n = 4), no significant difference was found between the two groups (figure 10). Thus, we were unable to determine a cutoff as the minimum PB-CD34+ cell purity required for production of megakaryocytes. This creates a limitation to our megakaryocyte culturing method since there is no assurance that certain CD34+ cell purity is useful for megakaryocyte culture set up. However, it was observed that PB-CD34+ cells from three donors consistently produced sufficient megakaryocytes when tested in three different experiments. Therefore, it may be possible to select a number of donors as “good donors” for megakaryocyte cultures in order to ensure the successful growth of megakaryocytes.

4.6 Future directions:

The megakaryocyte culture developed in this study has broad applications in the investigation of platelet disorders, drug discovery and in understanding the physiology of human megakaryopoiesis and platelet production.

Since this culture was optimized for ITP studies, it can be applied to investigate the pathogenic effects of ITP antibodies at all stages of

megakaryocyte growth and maturation. Previous studies have suggested that the presence of anti-CD42 and/or anti-CD41/61 impairs megakaryopoiesis (Chang et al., 2003, McMillan et al., 2004); however, the presence of other antibodies such as anti-c-Mpl (anti-CD110) have also been indicated in ITP (Kuwana et al., 2002). This megakaryocyte culture offers a functional assay for the identification and investigation of pathologic antibodies such as anti-CD110 in ITP.

This megakaryocyte culture can also be applied to investigate intrinsic disorders in patients with abnormal vWRF, *MYH9* related disease and myeloproliferative neoplasm. TPO receptor agonist drugs have been recently used with great success as a second line of treatment in ITP (Arnold et al., 2009); however it is not clear how these drugs restore megakaryopoiesis in the presence of ITP autoantibodies. The megakaryocyte culture developed in this study allows for the investigation of the restoring mechanism of TPO receptor agonist drugs in the presence of ITP antibodies and therefore contributes to the understanding of ITP and development of more effective drugs for this autoimmune disease.

REFERENCES

American Society of Hematology (2013) image 00001186 (image bank). Retrieved from http://imagebank.hematology.org

Arnold DM, Nazi I, Kelton JG, New treatments for idiopathic thrombocytopenic purpura: rethinking old hypotheses, *Expert Opinion on Investigational Drugs*, 2009, 18:805-819

Balduini A, Malara A, Pecci A, Badalucco S, Bozzi V, Pallotta I, Noris P, Torti M, Balduini CL. (2009) Proplatelet formation in heterozygous Bernard-Soulier syndrome type Bolzano. *Journal of thrombosis and hemostasis*; 7(3):478-84

Barnett D, Janossy G, Lubenko A, Matutes E, Newland A, Reilly JT(1999) Guideline for the flow cytometric enumeration of CD34+ haematopoietic stem cells (Prepared by the CD34+ haematopoietic stem cell working party. General Haematology Task Force of the British Committee for Standards in Haematology), *Clinical Laboratory Haematology*; 21(5):301-8

Boyer L, Robert A, Proulx C, Pineault N (2008) Increased production of megakaryocytes near purity from cord blood CD34+ cells using a short two-phase culture system, *Journal of immunological methods*; 332(1-2):82-91

Broudy VC, Lin NL, Kaushansky K. (1995) Thrombopoietin (c-mpl ligand) acts synergistically with erythropoietin, stem cell factor, and interleukin-11 to enhance murine megakaryocyte colony growth and increases megakaryocyte ploidy in vitro. *Blood*; 85(7):1719-26

Bunting S, Widmer R, Lipari T, Rangell L, Steinmetz H, Carver-Moore K, Moore MW, Keller GA, de Sauvage FJ (1997) Normal platelets and megakaryocytes are produced in vivo in the absence of thrombopoietin. *Blood*; 90(9):3423-3429

Chang M, Nakagawa PA, Williams SA, Schwartz MR, Imfeld KL, Buzby JS, Nugent DJ (2003) Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro, *Blood*; 102:887-895

Chen TW, Hwang SM, Chu IM, Hsu SC, Hsieh TB, Yao CL. (2009) Characterization and transplantation of induced megakaryocytes from hematopoietic stem cells for rapid platelet recovery by a two-step serum-free procedure. *Experimental Hematology*; 37(11):1330-1339

Chen TW, Yao CL, Chu IM, Chuang TL, Hsieh TB, Hwang SM (2009) Large generation of megakaryocytes from serum-free expanded human CD34+ cells. *Biochemical and biophysical research communications*; 378(1):112-7

Cho SH, Chung IJ, Lee JJ, Park ML, Kim HJ (1999) Comparison of CD34+ subsets and clonogenicity in human bone marrow, granulocyte colony-stimulating factor-mobilized peripheral blood, and cord blood. *Journal of Korean Medical Science*; 14(5):520-5

Cohen KS, Cheng S, Larson MG, Cupples LA, McCabe EL, Wang YA, Ngwa JS, Martin RP, Klein RJ, Hashmi B, Ge Y, O'Donnell CJ, Vasan RS, Shaw SY, Wang TJ (2013) Circulating CD34 (+) progenitor cell frequency is associated with clinical and genetic factors. *Blood*; 121(8): 50-56

Cortin V, Garnier A, Pineault N, Lemieux R, Boyer L, Proulx C (2005) Efficient in vitro megakaryocyte maturation using cytokine cocktails optimized by statistical experimental design. *Experimental hematology*; 33(10):1182-91

Crapnell K, Zanjani ED, Chaudhuri A, Ascensao JL, St Jeor S, Maciejewski JP (2000) In vitro infection of megakaryocytes and their precursors by human cytomegalovirus. *Blood*; 95:487-493

Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Seifert RA, Bowen-Pope DF (2000) Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. *Circulation Research*; 87(9):728-30

De Bruyn C, Delforge A, Martiat P, Bron D. (2005) Ex vivo expansion of megakaryocyte progenitor cells: cord blood versus mobilized peripheral blood, *Stem cell development*; 14(4):415-24

de Sauvage FJ, Hass PE, Spencer SD, Malloy BE, Gurney AL, Spencer SA, Darbonne WC, Henzel WJ, Wong SC, Kuang WJ, et al (1994) Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand, *Nature*; 369(6481), 533-538

Debili N, Wendling F, Katz A, Guichard J, Breton-Gorius J, Hunt P, Vainchenker W. (1995) The Mpl-ligand or thrombopoietin or megakaryocyte growth and differentiative factor has both direct proliferative and differentiative activities on human megakaryocyte progenitors, *Blood*; 86(7):2516-25

Deutsch VR, Tomer A (2013) Advances in megakaryocytopoiesis and thrombopoiesis: from bench to bedside, *British Journal of Haematology*; 161(6):778-93

Dolzhanskiy A, Hirst J, Basch RS, Karpatkin S (1998) Complementary and antagonistic effects of IL-3 in the early development of human megakaryocytes in culture. *British journal of hematology*; 100(2):415-26

Dolzhanskiy A, Basch RS, Karpatkin S (1997) The development of human megakaryocytes: III. Development of mature megakaryocytes from highly purified committed progenitors in synthetic culture media and inhibition of thrombopoietin-induced polyploidization by interleukin-3, *Blood*; 89:426-234

Drayer AL, Sibinga CT, Blom NR, De Wolf JT, Vellenga E (2000) The in vitro effects of cytokines on expansion and migration of megakaryocyte progenitors, *British journal of hematology*; 109(4):776-84

Egeland T. (1998) The CD34 molecule and hematopoietic progenitor cell studies--a challenge in clinical medicine. *Vox sanguinis*; 74 Suppl 2:467-8.

Fielder PJ, Gurney AL, Stefanich E, Marian M, Moore MW, Carver-Moore K, de Sauvage FJ (1996) Regulation of thrombopoietin levels by c-mpl-mediated binding to platelets. *Blood*; 87(6):2154-61

Fritsch G, Stimpfl M, Kurz M, Leitner A, Printz D, Buchinger P, Hoecker P, Gadner H (1995) Characterization of hematopoietic stem cells. *Annals of the New York Academy of Sciences*, 29; 770:42-52

Geddis AE. (2010) Megakaryopoiesis, *Seminars in hematology*; 47(3):212-219

Gerber JM, Smith BD, Ngwang B, Zhang H, Vala MS, Morsberger L, Galkin S, Collector MI, Perkins B, Levis MJ, Griffin CA, Sharkis SJ, Borowitz MJ, Karp JE, Jones RJ (2012) A clinically relevant population of leukemic CD34(+)CD38(-) cells in acute myeloid leukemia. *Blood*; 119(15):3571-7

Guerriero R, Testa U, Gabbianelli M, Mattia G, Montesoro E, Macioce G, Pace A, Ziegler B, Hassan HJ, Peschle C (1995) Unilineage megakaryocytic proliferation and differentiation of purified hematopoietic progenitors in serum-free liquid culture. *Blood*; 86(10):3725-36

Graham, J. M (2002), Isolation of platelets (Thrombocytes), *The scientific world journal*; 2: 1607-1609

Harmening, D.H (2005) Modern Blood Banking and Transfusion practices (5th ed.) F.A. Davis Company

Hoffman R, Murrav LJ, Young JC, Luens KM, Bruno E (1996) Hierarchical structure of human megakaryocyte progenitor cells, *Stem Cells*; 14 Suppl 1:75-81

Houwerzijl EJ, Blom NR, van der Want JJ, Esselink MT, Koornstra JJ, Smit JW, Louwes H, Vellenga E, de Wolf JT (2004) Ultrastructural study shows morphologic features of apoptosis and para-apoptosis in megakaryocytes from patients with idiopathic thrombocytopenic purpura, *Blood*, 103: 500-506

Invitrogen (2006), Propidium iodide nucleic acid stain data sheet, www.invitrogen.com

Invitrogen (2001), StemPro-34® SFM complete medium data sheet, www.invitrogen.com

Ishibashi T, Miller SL, Burstein SA (1987) Type beta transforming growth factor is a potent inhibitor of murine megakaryocytopoiesis *in vitro*, *Blood*; 69(6):1737-41

Italiano JE Jr, Lecine P, Shivdasani RA, Hartwig JH (1999) Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *The Journal of Cell Biology*; 147(6):1299-312

Italiano JE Jr, Shivdasani RA (2003) Megakaryocytes and beyond: the birth of platelets. Journal of Thrombosis and Haemostasis; 1(6):1174-1182

Kaushansky K (2002) Thrombopoietin: from theory to reality. *International Journal of Hematology*; 76 Suppl 1:343-345

Kaushansky K (1999) The enigmatic megakaryocyte gradually reveals its secrets, *Bioessays*; 21(4):353-60

Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Bailey MC, Forstrom JW, Buddle MM, Oort PJ, Hagen FS, et al. (1994) Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature*; 369(6481):568-71

Kuter DJ, Beeler DL, Rosenberg RD (1994) The purification of megapoietin: a physiological regulator of megakaryocyte growth and platelet production, *Proceedings of the National Academy of Sciences*; 91(23):11104-8

Kuwaki T, Hagiwara T, Yuki C, Kodama I, Kato T, Miyazaki H (1998) Quantitative analysis of thrombopoietin receptors on human megakaryocytes. *FEBS letters*; 427(1):46-50.

Kuwana M, Okazaki Y, Kajihara M, Kaburaki J, Miyazaki H, Kawakami Y, Ikeda Y. (2002) Autoantibody to c-Mpl (thrombopoietin receptor) in systemic lupus erythematosus: relationship to thrombocytopenia with megakaryocytic hypoplasia. *Arthritis Rheumatis*; 46(8):2148-59.

Lanza F, Moretti S, Castagnari B, Montanelli F, Latorraca A, Ferrari L, Bardi A, Dominici M, Campioni D, Dabusti M, Piva N, Lodi G, Reverberi R, Castoldi G (1999) Assessment of distribution of CD34 epitope classes in fresh and cryopreserved peripheral blood progenitor cells and acute myeloid leukemic blasts, *Haematologica* ; 84(11):969-77

Lepage A, Leboeuf M, Cazenave JP, de la Salle C, Lanza F, Uzan G. (2000) The alpha(IIb)beta(3) integrin and GPIb-V-IX complex identify distinct stages in the maturation of CD34(+) cord blood cells to megakaryocytes, *Blood*; 96:4169-4177

Leysi-Derilou Y, Robert A, Duchesne C, Garnier A, Boyer L, Pineault N. (2010) Polyploid megakaryocytes can complete cytokinesis. *Cell Cycle*; 9(13):2589-99

Majka M, Baj-Krzyworzeka M, Kijowski J, Reca R, Ratajczak J, Ratajczak MZ (2001) In vitro expansion of human megakaryocytes as a tool for studying megakaryocytic development and function, *Platelets*; 12 (6): 325-332

Mattia G, Vulcano F, Milazzo L, Barca A, Macioce G, Giampaolo A, Hassan HJ (2002) Different ploidy levels of megakaryocytes generated from peripheral or cord blood CD34+ cells are correlated with different levels of platelet release, *Blood*; 99:888-897

McMillan R, Wang L, Tomer A, Nichol J, Pistillo J (2004) Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP, ***Blood***; 103:1364-1369

Mehrishi JN, Bakács T (2013) A novel method of CD34+ cell separation from umbilical cord blood, *Transfusion*; ahead of print

Miltenyi biotech GmbH (2007), LS Columns data sheet, www.miltenyibiotec.com

Motabi IH, DiPersio JF (2012) Advances in stem cell mobilization, *Blood reviews*; 26(6):267-78

Nurden P, Gobbi G, Nurden A, Enouf J, Youlyouz-Marfak I, Carubbi C, La Marca S, Punzo M, Baronciani L, De Marco L, Vitale M, Federici AB (2010) Abnormal VWF modifies megakaryocytopoiesis: studies of platelets and megakaryocyte cultures from patients with von Willebrand disease type 2B. *Blood*; 115(13):2649-56

Oran B, Shpall E (2012) Umbilical cord blood transplantation: a maturing technology. American Society of Hematology Education Book; doi: 10.1182/asheducation-2012.1.215.

Pecci A, Malara A, Badalucco S, Bozzi V, Torti M, Balduini CL, Balduini A. (2009) Megakaryocytes of patients with MYH9-related thrombocytopenia present an altered proplatelet formation, *Thrombosis and hemostasis*; 102(1):90-6

Perdomo J, Yan F, Ahmadi Z, Jiang XM, Stocker R, Chong BH (2011) Quinine-induced thrombocytopenia: drug-dependent GPIb/IX antibodies inhibit megakaryocyte and proplatelet production in vitro, *Blood*; 117(22):5975-86

Provan D, Stasi R, Newland AC, Blanchette VS, Bolton-Maggs P, Bussel JB, Chong BH, Cines DB, Gernsheimer TB, Godeau B, Grainger J, Greer I, Hunt BJ, Imbach PA, Lyons G, McMillan R, Rodeghiero F, Sanz MA, Tarantino M, Watson S, Young J, Kuter DJ. (2010) International consensus report on the investigation and management of primary immune thrombocytopenia, *Blood*; 115: 168-186

Rodak, B., Fritsma, G. A., Doig, K. (2007). Hematology: the clinical principles and applications (3rd ed.) Missouri, Saunders Elsevier

Saito H (1997) Megakaryocytic cell lines, *Baillieres Clinical Haematology*; 10(1):47-63

Saur SJ, Sangkhae V, Geddis AE, Kaushansky K, Hitchcock IS (2010) Ubiquitination and degradation of the thrombopoietin receptor c-Mpl. *Blood*; 115(6):1254-63

Schipper LF, Brand A, Reniers N, Melief CJ, Willemze R, Fibbe WE. (2003) Differential maturation of megakaryocyte progenitor cells from cord blood and mobilized peripheral blood, *Experimental Hematology*; 31(4):324-30

Schulze H, Korpal M, Hurov J, Kim SW, Zhang J, Cantley LC, Graf T, Shivdasani RA (2006) Characterization of the megakaryocyte demarcation membrane system and its role in thrombopoiesis, *Blood*; 107(10): 3868–3875

Sutherland DR, Marsh JC, Davidson J, Baker MA, Keating A, Mellors A (1992) Differential sensitivity of CD34 epitopes to cleavage by Pasteurella haemolytica

glycoprotease: implications for purification of CD34-positive progenitor cells. *Experimental Hematology*; 20(5):590-599.

StemCell Technologies (2009) StemSep human CD34 selection cocktail procedure, www.stemcell.com

StemCell Technologies (2008) BIT 9500 Serum substitute data sheet, www.stemcell.com

Veljkovic DK, Rivard GE, Diamandis M, Blavignac J, Cramer-Bordé EM, Hayward CP. (2009) Increased expression of urokinase plasminogen activator in Quebec platelet disorder is linked to megakaryocyte differentiation, *Blood*; 113(7):1535-42

Wognum AW, Eaves AC, Thomas TE. (2003) Identification and isolation of hematopoietic stem cells, *Archives of Medical Research*; 34(6):461-75

Yang L, Wang L, Zhao CH, Zhu XJ, Hou Y, Jun P, Hou M.(2010), Contributions of TRAIL-mediated megakaryocyte apoptosis to impaired megakaryocyte and platelet production in immune thrombocytopenia. *Blood*; 116(20):4307-16

Yasui K, Matsumoto K, Hirayama F, Tani Y, Nakano T. (2003) Differences between peripheral blood and cord blood in the kinetics of lineage-restricted hematopoietic cells: implications for delayed platelet recovery following cord blood transplantation. *Stem Cell*; 21(2):143-51

Zeigler FC, de Sauvage F, Widmer HR, Keller GA, Donahue C, Schreiber RD, Malloy B, Hass P, Eaton D, Matthews W (1994) In vitro megakaryocytopoietic and thrombopoietic activity of c-mpl ligand (TPO) on purified murine hematopoietic stem cells. *Blood*; 84(12):4045-52.

Zimmet J, Ravid K, Polyploidy: occurrence in nature, mechanisms, and significance for the megakaryocyte-platelet system, Experimental Hematology, 2000, 28:3-16

Zeuner A, Signore M, Martinetti D, Bartucci M, Peschle C, De Maria R (2007) Chemotherapy-induced thrombocytopenia derives from the selective death of megakaryocyte progenitors and can be rescued by stem cell factor, *Cancer Research*; 67(10):4767-73