CYTOTOXIC T CELLS IN IMMUNE THROMBOCYTOPENIA

DEVELOPING A CYTOTOXIC T CELL ASSAY TO INVESTIGATE A $CD8^+$ T CELL PATHOLOGY IN MEGAKARYOPOIESIS IN IMMUNE THROMBOCYTOPENIA

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ABSTRACT

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder, characterized by platelet destruction and/or underproduction. The pathophysiology is heterogeneous and can be mediated by autoantibodies and cytotoxic T lymphocytes (CTLs). While platelet destruction in ITP is well documented, there is little support for platelet underproduction due to the inhibition of megakaryocyte growth and considerably less support for CTL-mediated platelet underproduction. Our objective was to develop an assay that could test for CTL-mediated inhibition of megakaryocyte growth (megakaryopoiesis) in ITP, using healthy controls.

Peripheral blood from healthy donors was used to prepare hematopoietic stem and progenitor cells (HSPCs). These cells were expanded with StemSpan to culture a large number of megakaryocytes for the CTL assay. Our studies show that CTLs can be stimulated *in-vitro* using anti-CD3 antibodies and that they can be used after freezing and thawing. We also assessed CTL stimulation via peptide presentation, using viral peptides whom almost 100% of the general population have memory CTL specificity to, in order to activate a lower frequency of CTLs and to model levels of CTL activation in autoimmune disease. Both stimulants were found to stimulate CTLs in healthy donors with donor variability in the IFN-γ ELISpot.

The CTL assay was developed by co-culturing thrombopoietin (TPO) stimulated HSPCs with autologous CTLs for 7 days to observe inhibition of megakaryocyte growth. To induce CTL stimulation, CTLs were either incubated with anti-CD3 or HSPCs were incubated with viral peptides before co-culturing with CTLs. Results showed that while

viral peptides can be used as an internal control for the CTL assay, it could not serve as a positive control as inhibition was donor dependent. Inhibition of megakaryocyte growth in the presence of anti-CD3 stimulated CTLs was observed in all donors, validating its use as an appropriate positive control to study $CD8^+$ T cell pathophysiology in ITP.

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LIST OF ABBREVIATIONS AND SYMBOLS

ACD	Acid citrate dextrose
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
APC	Antigen presenting cell
AUX	Auxiliary
BIT	Bovine serum albumin, insulin and transferrin
BSA	Bovine serum albumin
BM	Bone marrow
CD	Cluster of differentiation
CEF	Cytomegalovirus, Epstein Barr virus, Influenza virus
cMpl	Cellular myeloproliferative leukemia proto-oncogene
CMV	Cytomegalovirus
CTL	Cytotoxic T cell
DMSO	Dimethyl sulfoxide
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISpot	Enzyme linked immunospot
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Flt3L	Fetal 3 ligand

FS	Forward scatter
GP	Glycoprotein
HRP	Horseradish peroxidase
HSA	Human serum albumin
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IgG	Immunoglobulin G
IL-3	Interleukin 3
IL-6	Interleukin 6
IL-12	Interleukin 12
IL-2	Interleukin 2
IMDM	Iscove's Modified Dulbecco's Media
IFN-γ	Interferon gamma
ITP	Immune thrombocytopenia
IVIg	Intravenous immunoglobulins
KIR	Killer cell like immunoglobulin receptor
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MNC	Mononuclear cells
MK	Megakaryocytes
NK	Natural killer
NKT	Natural killer T

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PGE1	Prostaglandin E1
PE	Phycoerythrin
PE-CY5	Phycoerythrin-cyanine 5
REP	Rapid expansion protocol
PB	Peripheral blood
PRP	Platelet rich plasma
PVDF	Polyvinylidene difluoride
RPMI 1640	Roswell Park Memorial Institute 1640 medium
SCF	Stem cell factor
SS	Side scatter
SR1	StemReginin 1
TMB	3,3', 5,5'-Tetramethylbenzidine
TNF-α	Tumor necrosis factor - alpha
TPO	Thrombopoietin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

DECLARATION OF ACADEMIC ACHIEVEMENT

This study shows that peripheral blood derived $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) can be expanded to produce sufficient numbers of megakaryocytes for testing purposes. Megakaryocyte growth is inhibited in non-thrombocytopenic, healthy controls when HSPCs undergoing megakaryopoiesis are co-cultured with anti-CD3 stimulated cytotoxic T lymphocytes (CTLs). The number and percentage of cells expressing the CD41 surface antigen is decreased when HSPCs are co-cultured with CTLs and anti-CD3 compared to HSPCs alone or HSPCs co-cultured with CTLs. CTL stimulation due to viral peptide antigen and anti-CD3 incubation is not compromised after freezing. The assay can be carried out with peripheral blood (90 mL), yielding a minimum of 250,000 $CD34^+$ cells after expansion. Anti-CD3 induced CTL stimulation can serve as a model of CTL activation and CTL-mediated inhibition of megakaryocyte growth to study ITP.

The experiments outlined in this thesis were completed by Nadia Karim with some assistance. Rumi Clare previously assessed megakaryocyte/CTL co-culture assays, in a non-autologous system. Nikola Ivetic optimized the use of StemSep $CD34^+$ expansion supplement for peripheral blood $CD34^+$ cells and assisted with creating flow cytometry protocols for co-culture experiments.

1.0 INTRODUCTION

1.1 IMMUNE THROMBOCYTOPENIA

1.1.1 Definition, Clinical Diagnosis and Classification

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder with an incidence of 1 per 10,000 to 1 per 1000 persons per year (Psaila & Bussel, 2007). Individuals with ITP have low platelet numbers and normal to increased numbers of megakaryocytes (platelet precursor cells) in the bone marrow (Kuhne et al, 1998). ITP is a diagnosis of exclusion where a low platelet count and bleeding events are observed in the absence of any other attributable cause. Various laboratory tests are carried out to assess the presence of autoantibodies in ITP but they lack sensitivity and are not necessary or reliable for diagnosis. The lack of standard clinical testing in ITP remains to be one of the biggest problems in the field and is in part due to the heterogeneity in ITP pathophysiology. ITP guidelines published in 2009, describe a patient as having ITP when their platelet count is below 100 x 10^9/L (Neunert et al, 2011). Children with newly diagnosed ITP are often affected by the acute pathology and are often not subjected to medical intervention; many cases are resolved on the basis of observation alone. Adults usually present with chronic or persistent ITP and more females than males are affected (Arnold & Kelton, 2007).

ITP may be further characterized as primary or secondary. Primary or idiopathic ITP presents as a isolated low platelet count while secondary ITP arises as a result of another condition or infection such as HIV, Crohn's disease or hepatitis C (Arnold &

Kelton, 2007). ITP patients can present with purpura or bruising of the skin and smaller red lesions known as petechiae that appear on the skin or in the mouth, the latter, which may be indicative of mucocutaneous bleeding (Cohen et al, 2000). ITP patients with platelet counts greater than 30 x 10^9/L can experience mild mucocutaneous hemorrhages. When platelet counts fall below this threshold, clinically, patients are at risk for severe hemorrhaging (Neunert, 2011).

1.2 HETEROGENITY OF ITP PATHOPHYSIOLOGY

1.2.1 Autoantibodies in ITP

Clinicians have attempted to understand the mechanisms responsible for platelet destruction and/or underproduction in ITP for over 60 years (Ahn & Horstman, 2002). The earliest attempt to define the pathophysiology of ITP was made by Dr. William Harrington, in 1951, who observed a decreased platelet count after self-injection of ITP blood plasma (Harrington et al, 1951). Subsequent plasma infusion studies suggested that the drop in platelet numbers could be attributed to the presence of anti-platelet antibodies in ITP plasma (He et al, 1994). These antibodies are primarily of the IgG subtype and target glycoprotein (GP) IIb/IIIa and GP Ib/IX. Platelet coated antibodies are recognized by Fc gamma receptors on macrophages, engulfed and cleared by the reticuloendothelial system, which includes the spleen, liver and bone marrow (Podolanczuk et al, 2009). In one study, McMillian et al used an antigen capture assay to detect anti-platelet GP IIb/IIIa and Ib/IX antibodies in 282 chronic ITP patients (McMillian et al, 2003). The group found 56.4% of patients to have detectable antibodies, while a large number of patients had a negative test. Possible explanations for this finding included the presence of

alternate antibodies not tested for, prior treatment suppressing autoantibody activity and the presence of other immune mechanisms that could mediate platelet destruction and/or underproduction in ITP such as autoreactive T cells (McMillian, 2007).

1.2.2 Cytotoxic T lymphocytes in ITP

Several studies find a large number of ITP patients to have no detectable autoantibodies. These findings support the hypothesis for a heterogeneous pathophysiology where alternative immune mechanisms may be responsible for platelet destruction in ITP. Studies find ITP platelets to be targets for apoptosis by cytotoxic T lymphocytes (CTLs) characterized as CD8 positive ($CD8^+$) (Zhang et al, 2006). Platelet apoptosis was measured using a general apoptosis marker known as annexin-V – a protein that binds phosphatidyl serine, which becomes expressed on the surface of a cell during early apoptosis (Andree et al, 1990; Martin et al, 1995). Platelet apoptosis was significantly increased when platelets were incubated with autologous CTLs from ITP patients compared to non-thrombocytopenic controls (annexin-V labelled platelets: 7.56 ± 2.80% vs. 3.61 ± 0.90%) (Zhang et al, 2006). The caveat of this study was the use of annexin-V in isolation to investigate apoptosis, since platelets express PS both during apoptosis and activation (Dale, 2005). CTL proliferation has also been reported to be platelet specific where ITP CTL/platelet co-cultures yield a seven fold greater proliferation compared to healthy control samples (Li et al, 2007). Therefore, literature supports ITP platelet specific CTL activation and weakly supports CTL mediated platelet apoptosis.

CTL activity in ITP is also supported by increased levels of $CD8^+$ specific gene expression for proteins associated with cytotoxic activity. Studies reported active ITP patients to have increased $CD8^+$ T cell gene expression of Fas, granzyme A and B, perforin and IFN-γ compared to healthy controls (Olsson et al, 2003; Zhang et al, 2006). Recently, the cytotoxic granule protein, granzyme B was found to have a significant mechanistic role in CTL-mediated platelet destruction, where inhibition of granzyme B activity resulted in decreased ITP platelet apoptosis. Platelet apoptosis was not correlated to changes in other proteins such as granzyme A and perforin (Zhou et al, 2014). This research suggests that CTL activity in ITP may be granzyme B dependent and that granzyme B may be a potential marker for a $CD8^+$ T cell pathology in ITP.

While there is significant evidence for platelet destruction, there is minimal support for platelet underproduction in ITP as a result of megakaryocyte apoptosis or inhibition of growth. A single study carried out by Li and colleagues explored the impact of CTLs on megakaryocyte apoptosis in ITP patients using annexin-V (Li et al, 2007). The group initiated megakaryocyte cultures using bone marrow mononuclear cells and reported megakaryocyte apoptosis to be 16.58 ± 3.37% in ITP patients. Megakaryocyte apoptosis was increased (20.99 ± 4.21%) in ITP samples when megakaryopoiesis was initiated with mononuclear cells depleted of CTLs. Bcl-x_L – a pro-survival protein required for megakaryocyte survival during platelet formation (Josefsson et al, 2011), expression on ITP megakaryocytes was also significantly increased when megakaryopoiesis was initiated in the absence of CTLs (Li et al, 2007). The presence of CTLs during ITP megakaryocyte development resulted in increased megakaryocyte

counts, decreased megakaryocyte apoptosis and ploidy and reduced platelet counts (Li et al, 2007). These findings suggest a role for CTLs in the inhibition of megakaryocyte development, resulting in platelet underproduction. While these conclusions are encouraging, these results have been reported in a single study and further investigation is required to support or disprove the methodologies used to measure CTL induced inhibition of megakaryocyte growth.

1.3 TREATMENT

Therapy aimed at reducing antibody mediated platelet destruction and/or underproduction provides a favorable response in only 50-70% of ITP patients (Mitchell & Bennett, 2013). This further supports ITP as a "mosaic" disorder with several underlying immune mediators. Currently, standard therapy typically follows a "staircase" approach in which treatments of least toxicity are initiated first followed by more invasive and toxic options (Arnold & Kelton, 2007). If platelet counts fail to stabilize, patients are introduced to more aggressive and toxic treatment options. Since the underlying pathophysiologies of ITP have not yet been defined and because there is a lack of standard platelet antibody testing (Warner et al, 1999), the "staircase" approach to therapy is still used. Therefore, it would be valuable to develop a test that can identify the presence of autoreactive CTL activity in ITP patients to both, gain a greater understanding of the immune mechanisms underlying this pathology and aid in providing more pathology specific treatment regimes. Treating each individual ITP case based on pathophysiology may result in improved rates of remission and reduced side effects (Arnold & Kelton, 2007).

1.3.1 First-Line and Second-Line Therapies in ITP

Treatment in ITP is usually recommended for patients whose platelet counts have fallen below 30 x 10^9/L or below 50 x 10^9/L accompanied by bleeding events (Brit. J. Haematol, 2003). First-line therapy is corticosteroid-based, usually prednisone. Approximately 80% of patients respond to this form of treatment and 20% achieve remission (Arnold & Kelton, 2007). If prednisone is ineffective, treatment may be supplemented with intravenous immunoglobulin G (IVIg) therapy. IVIg works by increasing plasma IgG, thereby decreasing the efficiency of the reticuloendothelial system and reducing clearance of antibody-coated platelets (Kelton et al, 1985). If platelet counts fail to stabilize, second-line therapies are considered and include splenectomy, TPO mimetic agents and drugs that target abnormal B and T cell activity (Arnold & Kelton, 2007).

The spleen is the site responsible for recycling antibody-coated platelets. By removing the spleen, platelets numbers increase due to reduced platelet clearance, resulting in stabilized counts in 60-70% of patients. Splenectomy offers the best outcomes for patients with chronic ITP (Arnold & Kelton, 2007). Alternative to a splenectomy are drugs such as rituximab that targets the CD20 receptor on B cells, which are lymphocytes responsible for the production of platelet autoantibodies (Stasi et al, 2001). Treatment with rituximab results in autoreactive B cell depletion and rescued platelet counts (Coopamah et al, 2003). Following a failed splenectomy, 60% of patients respond to rituximab and approximately 20% achieve complete remission (Arnold & Kelton, 2007). A variety of drugs aim to specifically address T cell dysfunction in ITP (Kappers-Klunne

& Van't Veer, 2001). One such drug is cyclosporin A, which reduces IL-2 production from $CD4^+$ T cells. Inhibition of this pathway indirectly inhibits B cell activity. Clinical studies have shown the benefits of combining cyclosporin A and prednisone after resistance to both corticosteroid therapy and splenectomy. Results find that the combination provides increased immunosuppression in patients compared to prednisone treatment alone (Kappers-Klunne & Van't Veer, 2001).

1.4 MEGAKARYOPOEISIS AND THROMBOPOEISIS

To study the impact of CTLs on platelet underproduction in ITP it is necessary to first understand how megakaryocytes are formed in the bone marrow, the specific processes megakaryocytes undergo to produce platelets in a non-diseased state and the markers used to characterize these cells *in-vitro*.

1.4.1 Megakaryocyte Development and Structure

The bone marrow is the site for blood cell production and is a source of hematopoietic stem cells (HSCs) (Deutsch & Tomer, 2006). During hematopoiesis, HSCs differentiate to form the common myeloid and common lymphoid progenitor cells. The common myeloid progenitor gives rise to the common megakaryocyte–erythroid progenitor, which is responsible for producing megakaryocyte progenitors. The process by which megakaryocytes arise from hematopoietic stem cells and mature is called megakaryopoiesis. Megakaryocyte progenitors develop in response to a hematopoietic ligand known as the cellular myeloproliferative leukemia proto-oncogene (cMpl) ligand or thrombopoietin (TPO), which is constantly synthesized by the liver (Kaushansky,

2006). cMpl or the TPO receptor is expressed on hematopoietic tissues, megakaryocytes and platelets (Deutsch & Tomer, 2006). During megakaryocyte development, TPO binds the cMpl receptor on megakaryocyte progenitors and promotes proliferation and maturation (Kaushansky, 2003).

As megakaryocyte progenitors proliferate, they undergo a special type of mitosis known as endoreduplication (Deutsch & Tomer, 2006). Endoreduplication is a process by which cells duplicate their nuclear content without undergoing cellular division. Megakaryocyte nuclear divisions, on average, span from 2n – 64n and may proceed to a maximum of 128n (Zimmet & Ravid, 2000). This specialized cell division increases the number of cytoskeletal proteins, granules and organelles required to form an invaginated membrane system (IMS) in the megakaryocyte (Schulze et al, 2006), which has the potential to grow up to 60 μm in diameter (Gewirtz, 1986). The IMS is directly continuous with the megakaryocyte plasma membrane and has a role in platelet production. Thus, megakaryocytes are structurally unique and are characterized as multinucleated (polyploid), granular and having large cytoplasmic mass.

1.4.2 Platelet Production

The invaginated membrane system (IMS) in mature megakaryocytes is created to initiate the release of proplatelets (Schulze et al, 2006). Proplatelets are long protrusions that bud off of the mature megakaryocyte cytoplasm and are deposited in the vascular sinusoids of the bone marrow. Proplatelets contain cytoskeletal filaments called microtubules, which act to transport secretory granules and platelet proteins such as fibrinogen and von Willebrand factor from the megakaryocyte to the proplatelet ends,

which become platelets (Blair & Flaumenhaft, 2009). The process by which platelets (thrombocytes) arise from proplatelets and are released into the blood stream is termed thrombopoiesis (Figure 1).

Platelets have a role in maintaining hemostasis, which is the balance between thrombosis and bleeding. Excessive clotting or bleeding characterizes platelet disorders, which may or may not be due to abnormalities in platelet function. Understanding regulated platelet production is a prerequisite to understanding the biological mechanisms underlying platelet disorders. A single megakaryocyte undergoing thrombopoiesis produces, on average, 1000-3000 platelets (Stenberg & Levin, 1989). Approximately 10^{11} platelets are produced daily (Kaushansky, 2005a) with a lifespan of 8 – 9 days (Kaushansky, 2008). In healthy individuals, steady state platelet production is regulated by serum TPO. When platelets are released into circulation via thrombopoiesis, serum TPO (unbound) binds the cMpl receptor on platelets and the complex is internalized. In platelets, TPO acts to lower the threshold of agonists required to induce platelet aggregation (Chen et al, 1995). During thrombocytopenia, a term used to describe a state of low platelet numbers, levels of unbound serum TPO increase and become free to bind to the cMpl receptor on hematopoietic stem and progenitor cells and megakaryocytes promoting megakaryopoiesis, endoreduplication and megakaryocyte maturation leading to increased platelet production (Houwerzijl et al, 2005). A study carried out by Mukai and colleagues observed platelet counts and serum TPO levels in an enzyme-linked immunosorbent assay (ELISA) in healthy controls, ITP patients and amegakaryocytic thrombocytopenic (AMT) patients (1995). Compared to ITP, AMT patients have low

numbers of megakaryocytes in the bone marrow resulting in low platelet numbers. In AMT, serum TPO levels are increased in comparison to healthy controls, while serum TPO levels in ITP are only slightly elevated. This data suggests that platelet production in ITP may occur at a normal rate, resulting in regular TPO clearance. These observations support the rapid platelet destruction hypothesis (Kuter, 1996).

1.4.3 Megakaryocyte and Platelet Surface Markers

As megakaryocyte progenitor cells develop from HSCs, they acquire the expression of receptor glycoproteins (GP) IIb/IIIa (CD41) and Ib/IX (CD42) (Tomer, 2004). The expression of these glycoproteins is maintained on platelets and is important for platelet function in hemostasis including platelet adhesion and aggregation. *In-vitro* studies use these glycoprotein receptors as markers for megakaryocyte maturity as they correlate with nuclear DNA mass. Von Willebrand factor (VWF) binding is a sensitive marker for megakaryocyte maturity and it is used to assess the timing of the expression of CD41 and CD42 (Tomer, 2004). While CD41 expression is acquired early in megakaryocyte development, CD42 is acquired later and at a slower rate. At a nuclear content of 16n, megakaryocytes express three times as much CD41 as CD42 (Tomer, 2004).

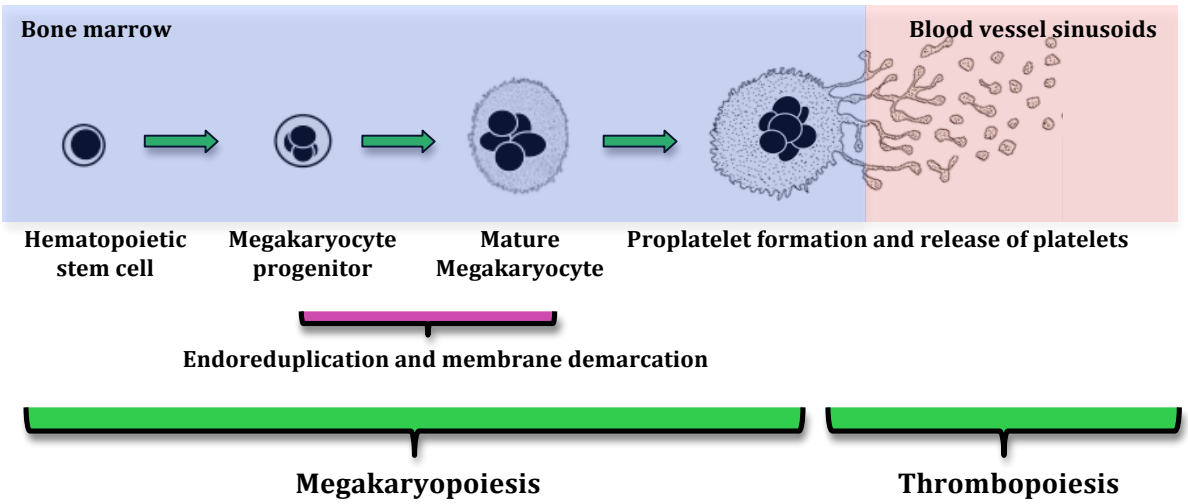


Figure 1 – Megakaryopoiesis and thrombopoiesis: A schematic showing the process of megakaryocyte development and platelet production. Megakaryopoiesis is the process by which mature megakaryocytes develop from hematopoietic stem cells in the bone marrow, via the stimulation of TPO. During development, megakaryocytes undergo endoreduplication in which the cell undergoes multiple nuclear divisions, without cytoplasmic divisions. Megakaryocytes develop a demarcated membrane to create membrane reserves to aid in platelet production. Thrombopoiesis describes the release of platelets into the bloodstream from proplatelet extensions derived from the megakaryocyte cytoplasm.

1.5 CULTURING MEGAKARYOCYTES IN-VITRO

Studying processes related to megakaryocyte biology and pathology is limited due to the technical barriers that exist in attaining large numbers of megakaryocytes (Majka et al, 2001). Technical barriers include the rarity of these cells in the bone marrow as well the availability of an efficient culture system to grow megakaryocytes *in-vitro*. The literature on *in-vitro* megakaryopoiesis consists of a variation of single stage early megakaryocyte expansion systems and two-stage hematopoietic stem cell (HSC)

expansion systems followed by megakaryopoiesis. While there are several published expansion systems for megakaryocyte progenitors (Majka et al, 2001; Proulx et al, 2003; Halle et al, 2000), in order to conduct studies on both mature megakaryocytes and the process of megakaryopoiesis it is necessary to find a system capable of expanding HSCs.

Hematopoietic stem cells, characterized as $CD34^+$, are expanded using a combination and variety of "classical" hematopoietic cytokines, developmental regulators and chemical modulators (Walasek et al, 2012). "Classical" hematopoietic cytokines consisting of stem cell factor (SCF), TPO, fetal 3 ligand (Flt3L), IL-11, IL-3 and IL-6 have previously been used in different combinations and doses to achieve HSC expansion. Recently, STEMCELL commercialized a "StemSpan CD34 Expansion Supplement" containing SCF, IL-3, 1L-6, Flt3L, TPO and other additives to support $CD34^+$ cell expansion. The supplement has been shown to expand human cord blood and bone marrow $CD34^+$ cells by > 10 fold in a 7-day period (STEMCELL Technologies, Vancouver, Canada). There are also a variety of chemical modulators that have been identified to play a role in HSC expansion such as StemReginin1 (SR1) discovered by Boitano et al (Boitano et al, 2010). SR1 is a synthetic molecule that antagonizes the aryl hydrocarbon receptor (AhR). SR1 blocks AhR transcriptional target genes and prevents the differentiation of early $CD34^+$ HSCs while promoting expansion of $CD34^+$ cells. Recent *in-vitro* studies support this finding and have reported that SR1 directly enhances HSC self-renewal in the presence of SCF, Flt3L, IL-6 and TPO (Csaszar et al, 2012). This combination of classical cytokines and a chemical modulator has been shown to successfully increase $CD34^+$ HSC yield from cord blood and mobilized peripheral blood

by 24-fold and 2.6-fold respectively in 1 week; and 670-fold and 73-fold respectively in 3 weeks (Boitano et al, 2010). While it is common practice to use these agents of HSC expansion to expand cord, bone marrow and mobilized peripheral blood derived HSCs, expansion of peripheral blood derived HSCs has not previously been done. Previous ITP studies regarding platelet production due to autoantibodies and cytotoxic T cells have cultured megakaryocytes from cord blood (Chang et al, 2003; Yang et al, 2010) and bone marrow derived HSCs (Li et al, 2007) respectively. In order to conduct studies requiring an autologous platform to study the interaction of megakaryocytes and cytotoxic T cells in ITP, it would be beneficial to assess the quality of these expansion systems on HSCs derived from peripheral blood – a more easily accessible source.

1.6 STUDYING CYTOTOXIC T CELLS IN AUTOIMMUNE DISEASE

Cytotoxic T cells play an integral role in adaptive immunity. They serve to recognize foreign peptides and directly kill infected cells via cytotoxic granule release. A variety of disorders including, but not limited to ITP have underlying cytotoxic T cell pathophysiologies. In order to understand the mechanisms involved in naïve and memory cytotoxic T cell activation, it is essential to study these cells *in-vitro*. This section will review T cell development and the various techniques used to activate cytotoxic T cells *in-vitro*.

1.6.1 T Cell Development

T cells are small lymphocytes that originate in the bone marrow and regulate the adaptive immune response. T cells mature in the thymus and are selected for release into

the peripheral blood to become apart of an individual's T cell repertoire, based on specificity and affinity for self peptides (Starr et al, 2003). During positive selection, T cells are introduced to major histocompatibility complex (MHC) receptors on cortical epithelial cells – specialized receptors that present peptides to T cells as a form of self-surveillance against foreign antigens (Starr et al, 2003).

MHC class I surface receptors are found on all nucleated cells and platelets while MHC class II receptors are found on antigen presenting cells (APCs) such as B cells, macrophages and dendritic cells (Starr et al, 2003). T cells interact with MHC molecules via their T cell receptors (TCRs). Double positive T cells ($CD4^+CD8^+$) with specificity to MHC class I are positively selected as $CD8^+$ T cells that develop a "killing" phenotype after activation. T cells that recognize MHC class II receptors are selected as $CD4^+$ T cells that develop a "helper" phenotype. Positive selection ensures that an individuals T cell repertoire is tolerant to self. Positively selected T cells that have a strong affinity or zero affinity to self-peptides and/or self-MHC complexes are eliminated in a process called negative selection. Negatively selected T cells undergo apoptosis due to macrophage promoted destruction and neglect respectively. T cells with strong affinity to self may contribute to autoimmune reactions and thus are negatively selected. After negative selection, the remaining T cells recognize self-peptides and MHC complexes with weak affinity and are released into circulation (Starr et al, 2003).

1.6.2 $CD8^+$ T Cell Activation and Effector Function

Naïve cytotoxic T cells ($CD8^+$) require antigen specific stimulation and essential cytokine signaling to become activated effector cells in a process known as priming

(Curtsinger et al, 2003). Peptide antigens are presented to $CD8^+$ T cells on MHC class I molecules, found on all nucleated cells, as well as platelets. Traditionally, MHC class I receptors present endogenous peptides to $CD8^+$ T cells but it is also possible that exogenous peptides can be presented to CTLs via a process known as cross-presentation (Wilson and Villadangos, 2005). During infection, dendritic cells present antigen to naïve $CD8^+$ T cells in secondary lymphoid organs. The T cell receptor (TCR) requires optimal binding to the peptide/MHC I complex. If the TCR has specificity for the complex, T cell motility decreases to facilitate dendritic cell interaction for increased periods of time. Dendritic cells provide a co-stimulation signal for T cells that triggers the T cell to release IL-2, stimulating its own proliferation and differentiation. Co-stimulation consists of the interaction between the CD28 receptor on T cells and the B7 ligands (CD80 and CD86) on dendritic cells. Co-stimulation is a necessary signal to prevent T cells from undergoing apoptosis or becoming anergic. Dendritic cells also release cytokines such IL-12 to enhance CTL specific activity and promote the release of effector specific cytokines (Curtsinger et al, 2003). Preceding activation, effector CTLs leave the lymphoid organs and migrate to infected tissues via chemokine signaling.

Cytotoxic T cells display killer effector function using two mechanisms – the perforin-granzyme pathway and the death receptor pathway (Kagi *et al*., 1994). The most common pathway used by $CD8^+$ T cells to kill their targets is via the release of perforin and granzyme. CTLs are composed of lytic granules that hold small proteins known as perforin and granzyme. Perforins specialize in pore formation while granzymes are serine proteases that promote apoptosis of target cells via the caspase pathway. Recent studies

conducted by Thiery et al suggest a two-step process for cytotoxic granule release (Thiery et al, 2011). The first step consists of the release of perforin and granzyme into the target cell by endocytosis into a specialized endosome – gigantosome. In the second step, perforin forms a pore in the gigantosome membrane triggering the release of cytosolic granzyme B into the target cell resulting in a caspase cascade. The death receptor pathway, although mechanistically distinct, achieves the same end point as granzyme release. This pathway consists of the interaction between the Fas ligand on the CTL with the death receptor on the target cell. Death receptors are part of the tumor necrosis factor (TNF) family of receptors that have a role in triggering the apoptotic signal via a similar caspase cascade (Hengartner, 2000).

1.6.3 Memory $CD8^+$ T Cells

After infection, a large number of activated $CD8^+$ T cells or short-lived effector cells (SLEC) undergo a phase of contraction (Parish & Kaech, 2009). The remaining memory precursor effector cells (MPEC) become part of the memory CTL population. Memory $CD8^+$ T cells can survive in absence of specific antigen for more than 50 years and are regulated in circulation by cytokine stimulation, specifically IL-15 and IL-17 (Hammarlund et al, 2003). During secondary infection, memory CTLs have faster effector responses due to high affinity to peptide antigen and increased frequency.

Previous *in-vitro* studies pertaining to memory T cell activation report that virus specific memory $CD8^+$ T cells only require TCR stimulation for activation (Bachmann et al, 1999). More recent *in-vivo* research in murine models has shown the requirement for CD28 co-stimulation for efficient memory CTL proliferation, whereby the absence of co-

stimulation results in a 9-fold expansion of CTLs compared to 40-fold expansion with co-stimulation (Fuse et al, 2008). The reduced proliferation in the absence of co-stimulation was determined to be the result of cell cycle arrest in the G1/S phase (Grayson et al, 2000; Chen et al, 2000). Murine models with antagonizing CD28 antibodies also showed increased viral loads, which prove the essential requirement for co-stimulation in addition to TCR stimulation to facilitate clearance of pathogens by memory CTLs (Grayson et al, 2000).

1.6.4 $CD8^+$ T Cell Activation *in-vitro*

There are several techniques used to activate $CD8^+$ T cells *in-vitro*. Studies find that the combination of immobilized anti-CD3 monoclonal antibody (mAb) (TCR stimulation) and anti-CD28 mAb (co-stimulation) results in increased T cell proliferation and decreased apoptosis compared to using the anti-CD3 mAb alone (Riddell and Greenberg, 1990). This method is useful in activating $CD4^+$ T cells, but less efficient for expanding and reducing apoptosis of $CD8^+$ T cells. A more reliable method for $CD8^+$ T cell activation is the rapid expansion protocol (REP), which consists of soluble anti-CD3 mAb and Fc gamma receptor (FcγR) bearing cells. The REP requires monocytes as accessory cells to provide FcγR that can bind the Fc region of immunoglobulin G antibodies. This results in increased anti-CD3 antibody presentation to T cells leading to increased TCR crosslinking (Clement et al, 1985). REP is most effective when $CD8^+$ T cells have been recently exposed to specific antigen *in-vivo* (Li & Kurlander, 2010).

1.6.5 Antigen Specific $CD8^+$ T Cell Activation *in-vitro*

Anti-CD3 antibodies induce TCR signaling without peptide antigen specificity. In disease, $CD8^+$ T cells are activated against specific peptide antigen. Additionally, autoreactive T cells exhibit low precursor frequencies and have low avidity for MHC/peptide complexes (Trudeau et al, 2003). To address this in our study, we aimed to find a peptide stimulant that would result in low frequency T cell activation similar to the level and specificity of T cell activation expected in autoimmune ITP. To model antigen specific $CD8^+$ T cell activation we used a commercial viral peptide pool, known as the CEF peptide pool (Currier et al, 2002). The pool consists of several peptide sequences from viruses that infect humans repeatedly over their lifetime, including cytomegalovirus (CMV), Epstein Barr virus (EBV) and influenza A. The prevalence of the CMV infection is approximately 60% by the age of 6 and almost 90% by the age of 80 in the general population (Staras et al, 2006). Similarly, the prevalence of the EBV memory T cell response is 95% in adults (Evans & Niederman, 1989) and almost 100% for influenza by the age of 12 (Sauerbrei et al, 2009).

The CEF peptide pool is frequently used as a positive control to activate antigen specific $CD8^+$ T cells. Autoimmune T cell reactivity is commonly measured using the IFN-γ ELISpot which can enumerate single T cell responses to peptide stimulation, previously seen in a study of $CD8^+$ T cells in autoimmune type I diabetes (Skowera et al, 2008). CEF peptides are useful in CTL assays because they only load onto MHC class I molecules and thus only elicit an immune response against $CD8^+$ T cells. Since every individual expresses a unique set of MHC alleles that recognizes and binds a unique set of

antigenic peptides (Nagy et al, 1989), the CEF pool, consisting of a total of 32 peptide sequences, provides a high likelihood that any given individual will elicit an immune response to at least one of the peptides present. In this study, the IFN-γ ELISpot was used to 1) validate the use of the CEF peptide pool as a positive control and 2) assess the impact of freeze-thaw on the ability of CTLs to respond to peptide stimulation. The ability to use functional $CD8^+$ T cells after freeze-thaw would increase efficacy in testing procedures and assay development.

2.0 RESEARCH OBJECTIVES

2.1 OBJECTIVES

2.1.1 Hypothesis

Cytotoxic T cells (CTLs) stimulated by common viral peptide presentation can serve as an appropriate positive control for CTL-mediated inhibition of megakaryocyte growth, which can act as a model to study ITP.

2.1.2 Primary Objective

Determine whether CTLs can inhibit megakaryocyte commitment and maturity, leading to platelet underproduction in ITP patients. To achieve this, we developed a model of CTL-mediated inhibition of megakaryocyte growth *in-vitro* using healthy controls.

2.1.3 Secondary Objectives

1. Optimize an expansion protocol for hematopoietic stem and progenitor cells, characterized as $CD34^+$, from minimal volumes of peripheral blood to overcome the limitation of low yield $CD34^+$ cells and ensure production of a sufficient number of megakaryocytes for our studies.
2. Validate the ability of CTLs to respond to peptide stimulation after freeze-thaw so that the assay can be carried out with a single blood draw.
3. Develop an *in-vitro* CTL assay to measure inhibition of megakaryocyte growth in healthy donors by stimulating a low frequency of CTLs in the presence of peripheral blood derived hematopoietic stem cells undergoing megakaryopoiesis.

3.0 METHODOLOGY

3.1 TISSUE CULTURE PROCEDURES

3.1.1 Preparation of Peripheral Blood Mononuclear Cells

Peripheral blood was collected from consented healthy donors into sterile anticoagulant dextrose (ACD) tubes. Peripheral blood mononuclear cells (PBMCs) were isolated by layering 25 mL of whole blood onto 15 mL of Histopaque 1077 (Sigma-Aldrich, St. Louis, USA) and tubes were centrifuged at 400 × *g* for 30 minutes. PBMCs were re-suspended in RPMI 1640 Medium (Life Technologies) (10% fetal bovine serum (FBS), penicillin/streptomycin and L-Glutamine). The first wash was supplemented with heparin (1 unit/mL) and centrifuged at 300 × *g* for 10 minutes. The second wash was carried out without heparin and centrifuged at the same speed and time. A 100 µL subsample was used to determine the cell count using the BECKMAN COULTER® Ac·T diff™ Analyzer. The BECKMAN COULTER® Ac·T diff™ Analyzer was used to determine all PBMC counts unless otherwise stated.

3.1.2 Preparation of $CD8^+$ T Cells From PBMCs

PBMCs were isolated from whole blood and a cell count was determined as described in *3.1.1*. PBMCs were centrifuged at 300 × *g* for 10 minutes and were resuspended in RoboSep buffer at a concentration of 1 x 10^8 cells/mL in a 14 mL polystyrene tube (BD Falcon 35-2057). The PBMC sample was loaded onto the RoboSep™ (STEMCELL Technologies, Vancouver, Canada) and the "human CD8 positive selection 18053 high purity" protocol was selected. The CD8 positive selection

EasySep kit (STEMCELL Technologies, Vancouver, Canada) including the magnetic nanoparticles, CD8 positive selection cocktail, RoboSep buffer bottle, flow through and waste collection tubes and the tip rack were subsequently loaded. Positive selection requires 50 minutes. Positively selected $CD8^+$ T cells were resuspended in 1 mL of RPMI and a cell count was determined using Trypan blue exclusion. During this process, PBMCs depleted of $CD8^+$ T cells were also attained.

3.1.3 Preparation of Platelets

Platelet rich plasma (PRP) was collected by centrifuging whole blood at 170 × *g* for 20 minutes. PRP was collected and centrifuged at 2000 × *g* for 10 minutes, followed by removal of plasma by aspiration. The platelet pellet was then resuspended in sterile filtered a mixture of calcium and albumin free (CAF) buffer and apyrase and a 100 µL subsample was used to determine the platelet count. Platelets were centrifuged at 2000 × *g* for 10 minutes before suspending pellet in RPMI media.

3.1.4 Preparation of Peripheral Blood $CD34^+$ Hematopoietic Stem and Progenitor Cells

Peripheral blood samples (90 mL) were collected from consented healthy donors into acid citrate dextrose tubes and processed under sterile conditions. Peripheral blood mononuclear cells (PBMCs) were isolated as described in *3.1.1* and resuspended in CD34 wash buffer (500 mL of phosphate buffered saline (PBS), 1% bovine serum albumin (BSA), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM theophylline, 0.15 µM aprotinin and 0.02 mM prostaglandin E1). An Opti-Prep density gradient reagent (Axis

Shield, Oslo, Norway) was used to remove excess platelets. PBMCs suspended in 25 mL of wash buffer were layered on top of 25 mL of Opti-Prep and centrifuged at 350 × *g* for 15 minutes. Supernatant was decanted and PBMCs were resuspended in 50 mL of wash buffer and centrifuged at 200 × *g* for 12 minutes. The PBMC pellet was re-suspended in CD34 wash buffer and a cell count was determined with a 200 μL PBMC subsample and the cell concentration was adjusted to 2 x 10^8 cells/mL. PBMCs were incubated with the StemSep™ Human CD34 Positive Selection Cocktail (STEMCELL Technologies, Vancouver, British Columbia) at 100 μl/mL of cells for 15 minutes at 4°C followed by incubation with the magnetic colloid at 60 μl/mL for 10 minutes at 4°C. PBMCs were washed with wash buffer and centrifuged at 370 × *g* for 10 minutes. The supernatant was removed and PBMCs were resuspended in 25 mL of wash buffer and $CD34^+$ cells were selected using the QuadroMACS™. Positively selected $CD34^+$ cells were centrifuged at 370 × *g* for 20 minutes and re-suspended in Iscove's modified Dulbecco culture medium – IMDM supplemented with 100 Units/mL penicillin and 100 μg/mL streptomycin and 15% bovine serum albumin (BSA), insulin and transferrin (BIT) Serum Substitute (STEMCELL Technologies). A subsample was used to determine the cell count using the TC20™ automated cell counter (BIO-RAD, Mississauga, Canada) and Trypan blue exclusion. To attain a cell count and viability, cells are mixed with a trypan blue stain at a 1:1 ratio. The stain only penetrates dead cells and cannot penetrate the intact membranes of live cells (Strober, 2001). All future cell counts for CD34 and megakaryocyte cultures were carried out using Trypan blue exclusion.

3.1.5 Expansion of Peripheral Blood CD34$^+$ Hematopoietic Stem and Progenitor Cells

3.1.5.1 StemReginin 1

Peripheral blood (90 mL) derived CD34$^+$ cells were seeded at 50,000 cells per well in 1 mL in IMDM supplemented with 100 Units/mL penicillin and 100 μg/mL streptomycin and 15% BIT 9500 serum in a 24-flat well bottom sterile culture plate (Corning, New York, USA). Cultures were supplemented with 100 ng/mL of each of the following cytokines TPO, SCF, IL-6, Flt3 and 1 uM of StemReginin 1 (SR1) (EMD Chemicals Inc. San Diego, CA) as specified in the expansion protocol in Boitano et al (2010). A DMSO control (0.01%), without SR1, was included to test for the impact of SR1 on CD34$^+$ cell expansion and self-renewal. Cultures were incubated at 37°C and 5% CO_2 and analyzed for expansion on day 6 and 12 via Trypan blue exclusion and flow cytometry for CD34 and CD41 expression. This combination of cytokines will be referred to as the StemReginin1 expansion system.

3.1.5.2 StemSpan CD34 Expansion Supplement

Peripheral blood (90 mL) derived CD34$^+$ cells were seeded at 50,000 cells per well in 1 mL of IMDM supplemented with 100 Units/mL penicillin and 100 μg/mL streptomycin and 15% BIT 9500 serum in a 24-well, flat well bottom sterile culture plate (Corning, New York, USA). StemSpan^{TM} CD34$^+$ Expansion Supplement (10X) *(STEMCELL Technologies, Vancouver, Canada),* containing SCF, IL-3, 1L-6, Flt3L, TPO and other additives, was added at 10% volume. Nikola Ivetic optimized the protocol for expansion of peripheral blood derived CD34$^+$ cells. Cultures were incubated at 37°C

and 5% CO_2 for 4 days. Cultures were analyzed on day 4 – Trypan blue exclusion and flow cytometry analysis were used to assess $CD34^+$ cell expansion and purity.

3.1.6 TPO Dose Response of Expanded $CD34^+$ Cells

3.1.6.1 Megakaryocytes Cultured From SR1 Expanded $CD34^+$ Cells

$CD34^+$ cell cultures expanded for 6 days with SR1 were centrifuged at 370 × *g* for 20 minutes to wash the culture free of expansion cytokines. $CD34^+$ cells were re-seeded at 50,000 cells/mL in 200 μL wells and supplemented with varying concentrations of TPO: 0, 10, 20 and 40 ng/mL in triplicates. TPO was added on day 0 and 6, post expansion. Cultures were analyzed on day 9, post expansion, using flow cytometry to assess megakaryocyte lineage commitment and maturity.

3.1.6.2 Megakaryocytes Cultured From StemSpan Expanded $CD34^+$ Cells

$CD34^+$ cells expanded for 4 days with StemSpan were centrifuged at 370 × *g* for 20 minutes to wash the culture free of expansion cytokines and re-seeded in IMDM media at 50,000 cells/well in 200 μL wells. TPO was added at varying concentrations: 0, 0.25, 0.50, 1, 10, 20 and 40 ng/mL and cells were cultured for 7 days. Cultures were analyzed on day 11 using flow cytometry to assess megakaryocyte lineage commitment and maturity.

3.1.7 Preparation of Viral Peptide Pool

The PepTivator CEF MHC Class I Plus – premium grade (Miltenyi Biotech Inc. San Diego, CA, U.S.A., Cat # 130-098-426) consists of a pool of 32 peptides at 6

nmol/peptide. The solid peptide pool was reconstituted with 200 µL of sterile water resulting in a stock concentration of 50 µg/mL of each peptide. 20 uL of the CEF peptide stock solution was added to 1 mL of cells at 1 x 10^8 cells/mL. Aliquots were stored at -20°C.

3.1.8 Cryopreservation of PBMCs

For cryopreservation, cells were resuspended in freezing medium, RPMI supplement with 10% dimethyl sulfoxide [DMSO; Sigma-Aldrich] and 12.5% human albumin serum at 1 x 10^7 cells/mL in 1 mL. Cells were transferred to 2 mL cryovials and frozen at -80°C in a Nalgene® Mr. Frosty container overnight and then transferred to liquid N_2 for long-term storage.

3.1.9 Thawing of Frozen PBMCs

For thawing, cryovials were removed from liquid N_2 storage and placed in a water bath at 37°C until the cell suspensions were almost thawed. The cells were transferred to 5 mL and then 8 mL of warm RPMI supplemented with heparin (1 unit/mL) was added drop wise. Cells were centrifuged at 350 × *g* for 10 minutes and the supernatant was decanted. Cells were resuspended in 1 mL of warm RPMI, counted and then plated at 5 x 10^6 cells/mL in a 24 well plate. Cells were incubated overnight at 37°C and 5% CO_2 atmosphere. Cells were pooled and counted the next day before use. The cells were then used in the IFN-γ ELISpot as previously described.

3.1.10 IFN-γ Enzyme-Linked ImmunoSpot (ELISpot)

An IFN-γ ELISpot assay (MABTECH 3420-2HW-Plus) was carried out with freshly isolated and cryopreserved PBMCs from peripheral blood (60 mL) to assess stimulation of T cells. Briefly, 96-well polyvinylidene fluoride (PVDF) plates (MAIP SWU10; Millipore, Bedford, MA) were activated with 50 μL of 70% ethanol for less than 2 minutes and then washed five times with sterile water (200 μL/well). Plates were coated with 100 μL/well of monoclonal anti-IFN-γ capture antibody (1-DIK, 15 μg/mL) (Mabtech 3420-2HW-Plus) in PBS and incubated overnight at 2-8°C. The plate was washed five times with PBS and blocked with RMPI supplemented with 10% FBS at 200 μL/well for 2 hours at 37°C. PBMCs, PBMCs depleted of $CD8^+$ T cells and $CD8^+$ T cells were placed into the wells in triplicate and incubated overnight with no peptide, anti-CD3 (mouse anti-human CD3 mAb, 1 μg/ml) or CEF peptides at a final concentration of 1 μg/mL of each peptide at 37°C and 5% CO_2. Cells were plated as follows: 100,000 PBMCs per well (20,000 for anti-CD3 stimulation), 75,000 PBMCs depleted of $CD8^+$ T cells per well (15,000 for anti-CD3 stimulation) and 25,000 $CD8^+$ T cells per well. The next day, cells were removed by five washes with PBS. The wells were then incubated with 100 μL of biotinylated anti-IFN-γ detector antibody (7-B6-1-Biotin; 1 μg/mL; Mabtech) for 2 hours at room temperature. The wells were then washed five times with PBS and incubated with 100 μL of streptavidin horseradish peroxidase (HRP) diluted 1:1000 in 0.5% BSA/PBS for 1 hour. Finally, the cells were washed five times with PBS and 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was added. The plate was developed for 10-15 minutes until distinct spots appear. Colour development

was stopped by washing the wells and the underside of the membrane with deionized water. The plate was inverted and allowed to dry in the dark at room temperature. When dry, the number of spots was quantified with the BioReader 5000 (BiSystems, GmbH). The number of spots quantified in the "anti-CD3" condition was multiplied by 5.

A second IFN-γ ELISpot was carried out with platelet/CTL co-cultures at a target/effector ratio of 1:100. The ELISpot plate conditions included platelets incubated with CEF peptides (1 μg/mL of each peptide), with CTLs, with CEF peptides and CTLs and with CTLs and anti-CD3 (1 μg/mL). All conditions were plated in triplicates. Platelets were incubated with CEF peptides for 1 hour prior to plating.

3.1.11 Megakaryocyte/CTL Co-culture Assays

3.1.11.1 Megakaryocyte Apoptosis Assay

Day 0

Peripheral blood samples (90 mL) were collected from healthy donors. $CD34^+$ HSPCs were positively isolated *(See 3.1.4)* and expanded with StemSpan expansion supplement for 4 days *(See 3.1.5.2)*. PBMCs that were not positively selected for were used to isolate autologous $CD8^+$ T cells and PBMCs depleted of $CD8^+$ T cells *(See 3.1.2)*. PBMCs, $CD8^+$ T cells and PBMCs depleted of $CD8^+$ T cells were cryopreserved the same day *(See 3.1.8)*.

Day 4

Expanded $CD34^+$ cells were centrifuged (washed free of expansion cytokines) and resuspended in fresh IMDM media supplemented with 100 Units/mL penicillin and 100 μg/mL streptomycin and 15% BIT 9500 serum as well as 20 ng/mL of TPO. Live $CD34^+$

cells were plated at 50,000 cells/well in 1 mL in a 24-well, flat well bottom plate and incubated for 7 days at 37°C and 5% CO_2 atmosphere.

Day 10

PBMCs, $CD8^+$ T cells and PBMCs depleted of $CD8^+$ T cells were thawed and rested overnight *(See 3.1.9)*.

Day 11

A subsample of TPO stimulated HSPCs was used to carry out a cell count using Trypan blue exclusion. Cultures were then analyzed on flow cytometry using FITC conjugated CD41 mAb and PE conjugated CD42 mAb. The number of live megakaryocytes ($CD41^+$ cells) was calculated by multiplying the live cell count by the percentage of cells expressing CD41. Live $CD41^+$ cells were adjusted to a concentration of 1 x 10^6 cells/mL.

Rested PBMCs, $CD8^+$ T cells and PBMCs depleted of $CD8^+$ T cells were centrifuged, resuspended in IMDM media, counted and diluted to a concentration of 2 x 10^6 cells/mL. $CD41^+$ cells were co-cultured with: PBMCs, PBMCs depleted of $CD8^+$ T cells and $CD8^+$ T cells (CTLs). $CD41^+$ cells were cultured alone, *"CD41"*, with CTLs, *"CD41 + CTLs"*, with CEF peptides (1 µg/mL of each peptide) and CTLs, *"CD41 + CEF + CTLs"* and with CTLs and anti-CD3 (5 µg/mL), *"CD41 + CTLs + anti-CD3."* Identical co-culture groups were set up for PBMCs and PBMCs depleted of $CD8^+$ T cell conditions. An additional condition was set up with $CD41^+$ cells alone, which were to be incubated with calcium ionophore on the day of flow cytometry analysis to induce maximum cell apoptosis, *"CD41 + Ca^{2+} ionophore"*. The target to effector ratio of

$CD41^+$ cells to PBMCs was 1:160. Ratios of $CD41^+$ cells to PBMCs depleted of $CD8^+$ T cells and to $CD8^+$ T cells alone were based on individual donor $CD8^+$ T cell percentages of PBMCs. $CD41^+$ cells were incubated with CEF peptides for 1 hour at room temperature (20 µL of CEF peptide pool mix per 1mL of cells [1 x 10^6 cells/mL] per manufacture's instructions) prior to co-culturing with CTLs. 10,000 live $CD41^+$ cells seeded for each condition in a 96-well micro titer plate, and co-cultured with CTLs at a ratio of 1:20 and 1:10. Experiments were performed in triplicated. Co-cultures were incubated overnight at 37°C and 5% CO_2 atmosphere.

Day 12

Co-cultures were assessed for $CD41^+$ cells co-expressing annexin-V using flow cytometry. *"CD41 + Ca^{2+} ionophore"* cultures were incubated with calcium ionophore (Sigma-Aldrich, St. Louis, USA), 20 minutes prior to performing flow cytometry. Calcium ionophore is a positive control for cell apoptosis. It facilitates the transport of calcium ions into the cell cytoplasm of intact cells and induces apoptosis via the intrinsic mitochondrial pathway (Mutlu, Gyulkhandanyan, Freedman and Leytin, 2012). Megakaryocyte apoptosis was measured by comparing percent of cells expressing CD41 surface antigen and cells expressing both CD41 and CD42 surface antigen in the "CD41" condition to all other conditions.

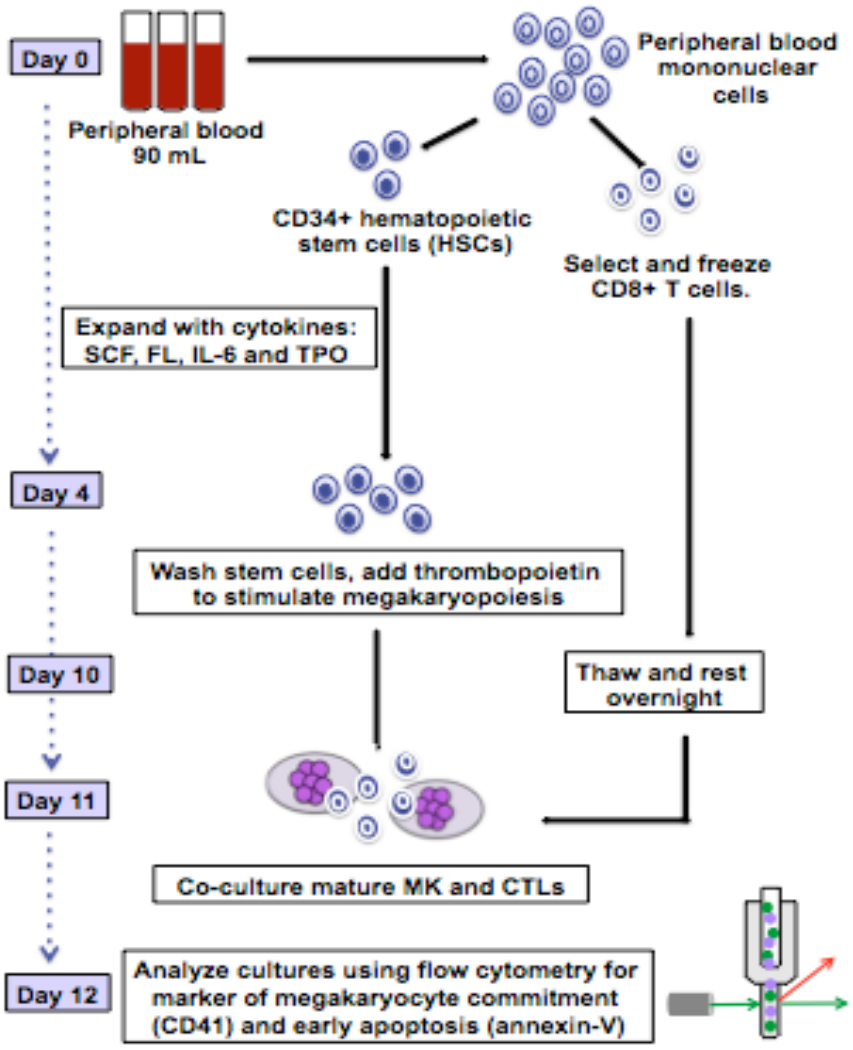


Figure 2 – Methods used to assess megakaryocyte apoptosis in the presence of healthy donor CTLs. On day 0, peripheral blood (90 mL) from healthy donors was used to isolate PBMCs and positively select for $CD34^+$ HSPCs and autologous CTLs ($CD8^+$). While CTLs were frozen, HSPCs were expanded using StemSpan cytokines. On day 4, HSPCs were centrifuged and then stimulated with TPO for 7 days. On day 10, CTLs were thawed and rested overnight. On day 11 megakaryocytes were co-cultured with CTLs either alone or in the presence of CEF peptides or anti-CD3. Note, CEF peptides were incubated with megakaryocytes prior to culturing with CTLs. Cultures were incubated overnight and analyzed on flow cytometry for annexin-V labeling on $CD41^+$ cells.

3.1.11.2 Megakaryopoiesis Growth Inhibition Assay

Day 0

Peripheral blood samples (90 mL) were collected from healthy donors. $CD34^+$ HSPCs were positively isolated *(See 3.1.4)* and expanded with StemSpan expansion supplement for 4 days *(See 3.1.5.2)*. PBMCs that were not positively selected for were used to isolate autologous $CD8^+$ T cells *(See 3.1.2)*. $CD8^+$ T cells were cryopreserved the same day *(See 3.1.8)*.

Day 3

$CD8^+$ T cells were thawed and rested overnight *(See 3.1.9)*.

Day 4

Expanded HSPCs were analyzed using flow cytometry, centrifuged, resuspended in IMDM media at 1 x 10^6 cells/mL and supplemented with 10 ng/mL of TPO. $CD8^+$ T cells were centrifuged and resuspended in IMDM at 2 x 10^6 cells/mL. $CD34^+$ HSPCs were cultured in 5 conditions: alone, with CTLs, with CTLs and CEF peptides (1 μg/mL of each peptide), with CTLs and anti-CD3 (5 μg/mL) and with CEF peptides. HSPCs were cultured with CEF peptides for one hour prior to co-culturing. $CD34^+$ cells (10,000) were plated for each condition in a 96 well flat-well plate and co-cultured with CTLs at ratios of 1:20, 1:10 and 1:5 (a target to effector ratio was settled at 1:10). Cultures were incubated for 7 days at 37°C and 5% CO_2 atmosphere.

Day 11

HSPC/CTL co-cultures were analyzed on day 11 using flow cytometry for megakaryocyte lineage commitment (CD41 expression) and maturity (CD42 expression).

Inhibition of megakaryocyte growth was measured in all conditions by comparing the percent of cells expressing CD41 and cells expressing both CD41 and CD42 to the level of expression seen in the condition with HSPCs stimulated with TPO in isolation.

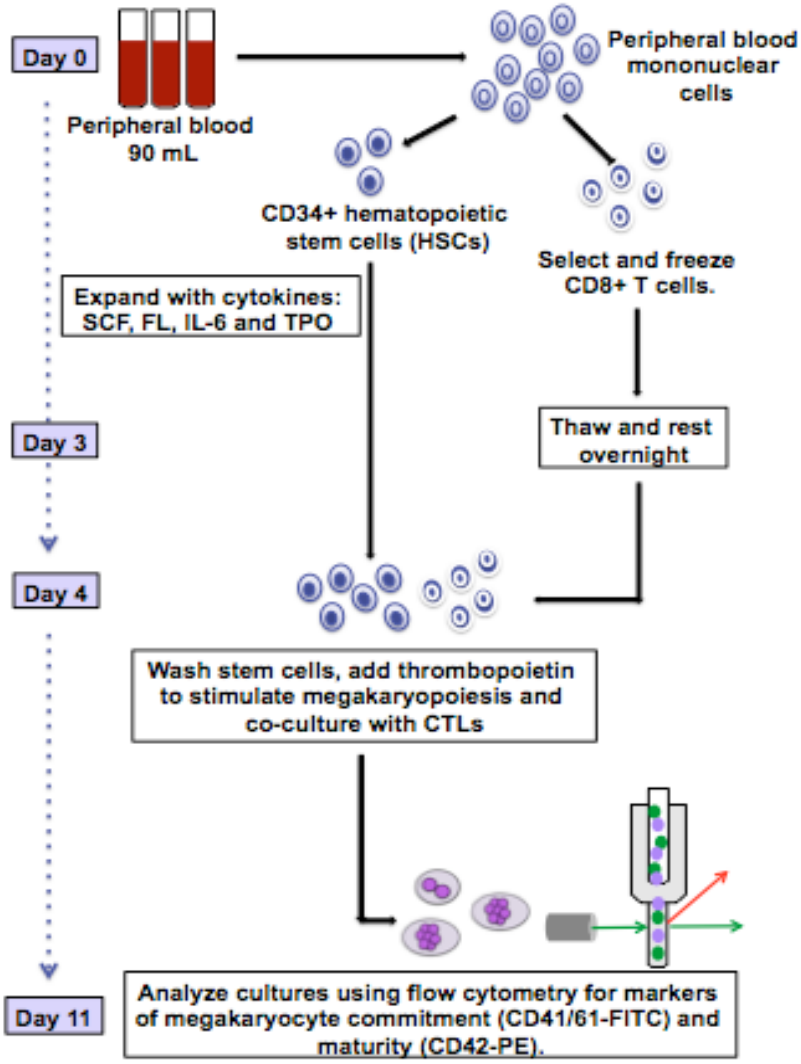


Figure 3 – Methods used to assess inhibition of megakaryocyte growth in the presence of healthy donor CTLs. On day 0, peripheral blood (90 mL) from healthy donors was used to isolate PBMCs and positively select for $CD34^{+}$ HSPCs and autologous CTLs ($CD8^{+}$). While CTLs were frozen, HSPCs were expanded using StemSpan. On day 3, CTLs were thawed and rested overnight. On day 4, HSPCs were centrifuged, stimulated with TPO and further co-cultured with CTLs either alone or in the presence of CEF peptides or anti-CD3. Note, CEF peptides were incubated with HSPCs prior to culturing with CTLs. Cultures were incubated for 7 days and analyzed on flow cytometry for CD41 and CD42 surface expression on megakaryocytes.

3.2 FLOW CYTOMETRY ANALYSIS

Flow cytometry was carried out using a four-channel flow cytometer (Beckmann Coulter Epics XL-MCL). FlowJo software (Tree Star, Inc. Ashland, OR, U.S.A) was used to complete analysis for all events collected during flow cytometry acquisition. All samples were stained with antibody bound fluorophore and incubated for 20 minutes at 4°C before conducting flow cytometry. Isotype matched control antibodies were used in each analysis and cells were stained so that the concentration of the isotype control antibody was equal to the concentration of the primary test antibody. Isotype controls were used to rule out non-specific Fc receptor binding as well as to confirm specific binding of the primary test antibody. Additionally, all experiments included compensation controls (a no colour control and single colour controls) to address the spectral overlap of one fluorophore into the fluorescence spectrum of another; compensation was accomplished manually using FlowJo. All monoclonal antibodies (mAb) used in this study were purchased from Becton Dickinson (BD) Biosciences (Mississauga, Canada).

3.2.1 $CD8^+$ Cytotoxic T cells

High purity $CD8^+$ T cell isolations using the RoboSep were confirmed using flow cytometry. $CD8^+$ T cells were characterized in our study as $CD45^+CD8^+$. The CD45 surface antigen is known as the leukocyte common antigen and is found on all hematopoietic cell types including lymphocytes, monocytes and granulocytes. By identifying cells that possess the CD45 antigen, platelets, red blood cells and debris are disregarded during signal acquisition (Barnett et al, 1999). CD8 T cell purity was

determined using a 100 µL sample labeled with fluorescein isothiocyanate (FITC) conjugated CD45 mAb and phycoerythrin (PE) conjugated CD8 mAb (RP8 clone). The purity of the $CD8^+$ T cell isolation was assessed as a percent of CD45 positive events that were also positive for CD8 by analyzing events using the FL1 (FITC) vs. FL2 (PE) (linear scale) dot plot.

3.2.2 $CD34^+$ Hematopoietic Stem and Progenitor Cells

Hematopoietic stem and progenitor cells **(**HSPCs) are characterized as $CD45^+CD34^+$. The CD34 antigen is a well-defined marker expressed on HSPCs. Samples (100 µL) were labeled with FITC conjugated CD45 mAb and PECy5 conjugated CD34 mAb, at a concentration of 1×10^5 cells/mL. The minimum number of events collected was 5,000. Events were analyzed using forward scatter (FS) vs. side scatter (SS) dot plot in linear scale (Figure 4). A gate was created around the lymphocyte population to exclude apoptotic cells and debris from analysis. During apoptosis, cells shrink and undergo chromatin condensation resulting in a reduction in FS and an increase in SS respectively (Darzynkiewicz et al, 1992). The lymphocyte population was then analyzed using the auxiliary (AUX) parameter vs. FS in a linear scale. AUX is a function that describes the height of the voltage pulse. A gate was created around singlet events as to exclude groups of cells that have clumped together, which may include both cells of interest and other cells, and have been recorded as a single large event. Next, a gate was created on events positive for CD45. The last step was to create a gate for CD34 positive events within the CD45 positive gate. The number of $CD34^+$ HSPCs isolated was determined [live cell count × % $CD45^+CD34^+$ events].

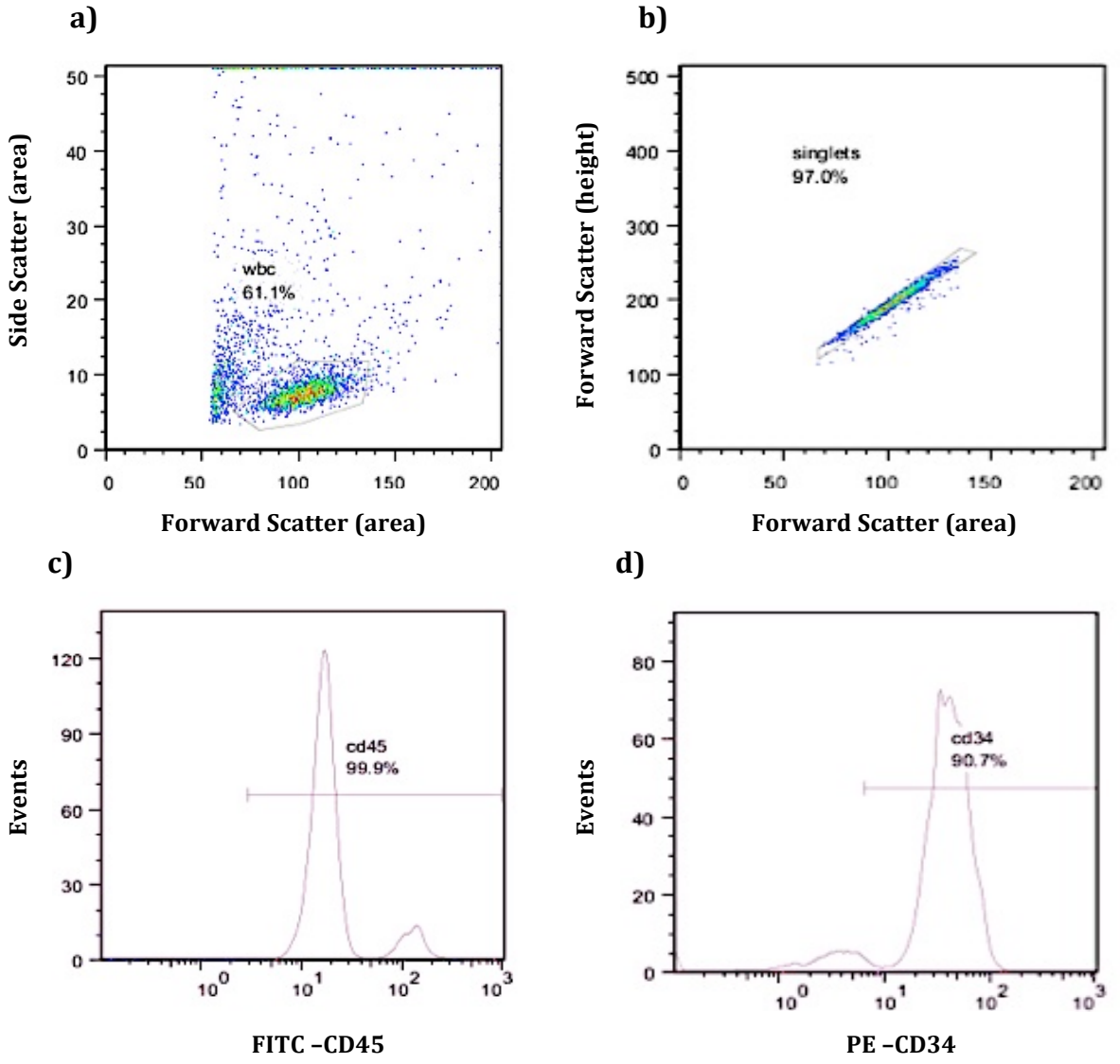


Figure 4 – Gating strategy for flow cytometry analysis of freshly isolated $CD45^{+}CD34^{+}$ hematopoietic stem and progenitor cells: The emission of FITC conjugated CD45 was detected in the FL1 channel and the emission of PE conjugated CD34 was detected in the FL4 channel. **a)** To assess $CD45^{+}CD34^{+}$ cell purity using Flow Jo software the lymphocyte population was gated in the linear scale, forward scatter (FS) (area) vs. side scatter (SS) (area); **b)** To remove doublets (cell clumping) from analysis and prevent decreased accuracy in purity measurement, singlet events were gated, using FS (area) vs. FS (height) linear; **c)** CD45 positive events were gates next to include all white blood cells; **d)** CD34 positive events were gated within the CD45 positive gate to determine the % of cells that were $CD45^{+}CD34^{+}$ HSPCs.

3.2.3 Megakaryocyte Lineage Commitment and Maturity

HSPCs committing to the megakaryocyte lineage ($CD41^+$) and mature megakaryocytes (double positive for both CD41 and CD42 surface antigen ($CD41^+CD42^+$)) were analyzed on flow cytometry using FITC conjugated CD41 mAb and PE conjugated CD42 mAb. A gate was created around the live cell population to exclude apoptotic cells and debris from analysis using the FSC and SSC parameters. A gate was created for CD41 positive events in the FSC (linear scale) vs. FL1 (log scale) dot plot. A gate of CD42 positive events was created in the CD41 positive events gate using FSC (linear scale) vs. FL2 (log scale) dot plot.

Megakaryocytes in co-culture with CTLs were assessed for CD41 and CD42 positive events using FITC conjugated CD41 mAb, PE conjugated CD42 mAb and PE-Cy5 conjugated CD8 mAb. PE-Cy5 conjugated CD8 mAb was used to exclude CD8 positive events from flow cytometry analysis for donors G, A upon re-testing and donor E during initial testing. The number of megakaryocytes in the HSPCs alone condition was determined [live cell count × % CD41]. The number of megakaryocytes in the megakaryocyte/CTL co-culture groups was determined [live cell count × % $CD8^-$ events × % $CD41^+$ events]. Following the exclusion of CD8 positive events from analysis, the number and percentage of cells $CD41^+$ and $CD41^+CD42^+$ in each group was determined. Prior to using a CD8 mAb, % CD41 could not be compared between the HSPCs alone condition and the HSPC/CTL co-culture conditions. In donors A, B and G an attempt was made to exclude $CD8^+$ events based on size in the FSC vs. SSC (linear scale) profile

using FlowJo. These results were accepted as true due to similar absolute number values for $CD41^+$ and $CD41^+CD42^+$ as seen before the exclusion of CD8 positive events.

3.2.4 Early Apoptotic Megakaryocytes

Early apoptotic megakaryocytes ($CD41^+$annexin-V^+) were analyzed on flow cytometry using FITC conjugated CD41 mAb and PE conjugated annexin-V. The sample tube was run dry to collect the maximum number of events. To assess the percentage of megakaryocytes that were annexin-V^+, platelets ($CD41^+$) were excluded from analysis, based on size, using the FSC vs. SSC in log scale. A CD41 single colour control was used to create a gate for annexin-V positive events. A gate was created around CD41 positive events using the FSC (linear scale) vs. FL1 (log scale) dot plot, followed by creating an annexin-V positive events gate within the CD41 positive events gate.

3.3 STATISTICAL ANALYSIS

Results are expressed as means ± SD and statistically analyzed by two-way ANOVA using GraphPad Prism (Version 6). The Tukey's multiple comparisons test was used to attain p values. A p value < 0.05 was determined to be statistically significant for all experiments.

4.0 RESULTS

4.1 EXPANDING CD34$^+$ HEMATOPOEITIC STEM AND PROGENITOR CELLS AND CULTURING MEGAKARYOCYTES FROM PERIPHERAL BLOOD

Our studies have shown that hematopoietic stem and progenitor cells (HSPCs) prepared from healthy donor peripheral blood in the laboratory, make up on average, 0.001% of peripheral blood mononuclear cells, after platelet depletion. To attain a sufficient number of HSPCs to use for culturing large numbers of megakaryocytes it was necessary to determine the best protocol for the expansion of CD34$^+$ HSPCs from peripheral blood. To accomplish this, we tested two different two-stage expansion systems. Both systems were previously used to expand CD34$^+$ cells from bone marrow and/or cord blood, but have not be assessed for their expansion of peripheral blood CD34$^+$ cells. Peripheral blood (PB) derived CD34$^+$ HSPCs were expanded by optimizing existing protocols in literature including Boitano et al (2010) and STEMCELL TECHNOLOGIES (2013) for the use of the StemReginin 1 (SR1) expansion system and StemSpan^{TM} CD34$^+$ Expansion Supplement (10X) respectively.

High purity PB derived CD34$^+$ HSPCs (> 90%) were prepared and expanded for 6 and 12 days using the SR1 expansion system. The percentage of HSPCs that were CD34$^+$ and the fold increase of CD34$^+$ cells following the expansion period was greater when expansion cultures were supplemented with SR1 (day 6: SR1 – 94.3% [4.01 fold increase] vs. DMSO control – 54.8% [1.56 fold increase]; day 12: SR1 – 74.6% [25 fold increase] vs. DMSO control – 14.3% [1.77 fold increase]) (Table I). On day 6, 37.6% of CD34$^+$ HSPCs expanded in the presence of SR1 were also CD41$^+$. These results

suggested that SR1 can aid in $CD34^+$ cell expansion but cannot prevent a subset of $CD34^+$ HSPCs from differentiating and committing to the megakaryocyte lineage.

SR1 expanded $CD34^+$ HSPCs were shown to be TPO dose dependent. Increased concentrations of TPO correlated to increased expression of the CD41 surface antigen in megakaryocyte cultures. HSPCs stimulated with 10 ng/mL of TPO for 9 days post expansion, resulted in maximal percent $CD41^+$ and $CD41^+CD42^+$ expression correlating with megakaryocyte lineage commitment and maturity respectively. In culture, 70% of cells were $CD41^+$ and 95% were $CD41^+CD42^+$ (Figure 5a). HSPCs stimulated with 20 ng/mL of TPO resulted in maximal $CD41^+$ and $CD41^+CD42^+$ megakaryocyte numbers (Figure 5b). Therefore, SR1 expanded PB $CD34^+$ HSPCs can be stimulated with 20 ng/mL of TPO to culture the maximal number of mature megakaryocytes.

Table I: Expansion of peripheral blood derived $CD34^+$ cells from healthy donors using the StemReginin1 (SR1) expansion system. Healthy donor $CD34^+$ HSPC cultures (n=1; donor A) were assessed for CD34 surface expression (% of total cells) and the fold increase in the absolute number of $CD34^+$ cells after 6 and 12 days of expansion. Cultures that were expanded with SR1 compared to DMSO control, resulted in greater percent CD34 purity and total cell number after expansion on both day 6 and 12. Cultures expanded without SR1 did not have increased expansion on day 12 compared to day 6.

	Days of Expansion			
	6		**12**	
	% CD34	**Fold increase of the absolute number of $CD34^+$ cells**	**% CD34**	**Fold increase of the absolute number of $CD34^+$ cells**
SR1 expansion system	94.3	4.01	74.6	25
DMSO control	54.8	1.56	14.3	1.77

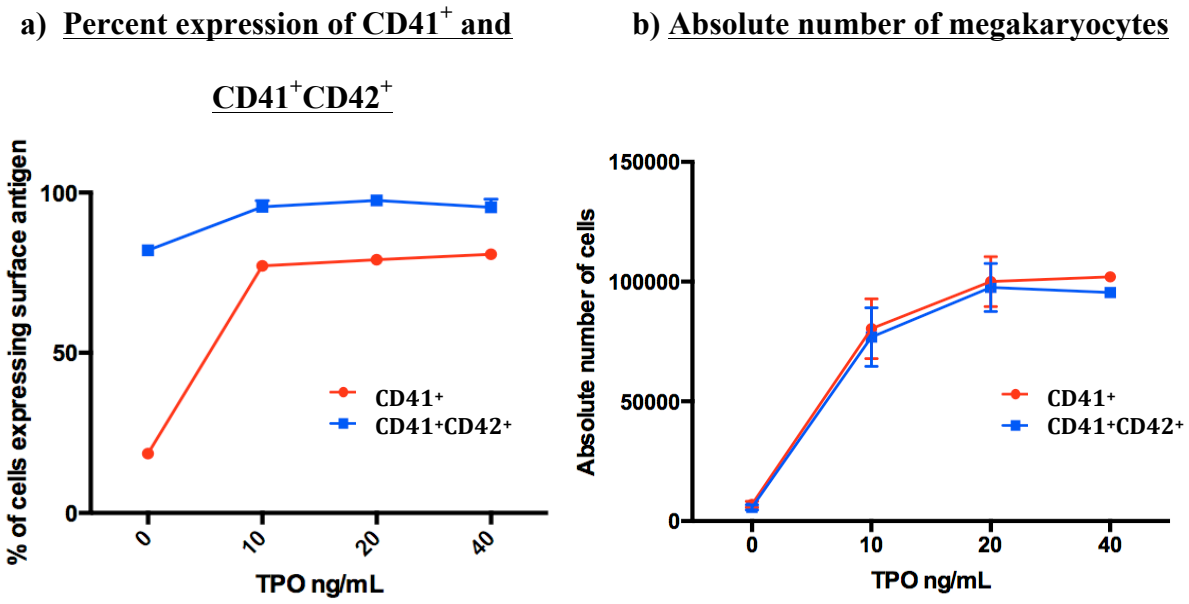


Figure 5 – Culturing megakaryocytes from CD34$^+$ cells expanded using the StemReginin 1 (SR1) expansion system. Expanded HSPCs (n=1; donor A) were stimulated with varying concentrations of TPO and were shown to be TPO dose dependent. HSPCs were stimulated with TPO on day 0 and 6, post expansion and were cultured for 9 days. **a)** The percent of total cells that were CD41$^+$ (red) and the percent of cells that were CD41$^+$CD42$^+$ (blue) are shown. TPO stimulation at 10 ng/mL resulted in the maximum percent of cells that were CD41$^+$ and CD41$^+$CD42$^+$; **b)** The absolute number of CD41$^+$ cells and CD41$^+$CD42$^+$ cells. TPO stimulation at 20 ng/mL resulted in the maximum number of cells that were CD41$^+$ and CD41$^+$CD42$^+$.

High purity PB derived CD34$^+$ HSPCs (> 90%) were also expanded with the "StemSpan^{TM} CD34 Expansion Supplement (10X)" (STEMCELL Technologies, Vancouver, Canada) containing SCF, IL-3, 1L-6, Flt3L, TPO and other additives. Nikola Ivetic optimized the number of days required for expanding peripheral blood derived CD34$^+$ HSPCs to be 4 days. The average CD34$^+$ HSPC expansion in 4 days with StemSpan was 8.73 ± 2.9 fold (n = 4). Individual donor CD34$^+$ HSPC fold expansion is summarized in Table II. CD34$^+$ HSPCs that were also CD41$^+$ on day 4 ranged between 8

and 25%. These results showed that StemSpan promotes high fold expansion of $CD34^+$ cells from peripheral blood, with minimal differentiation to other blood cell lineages.

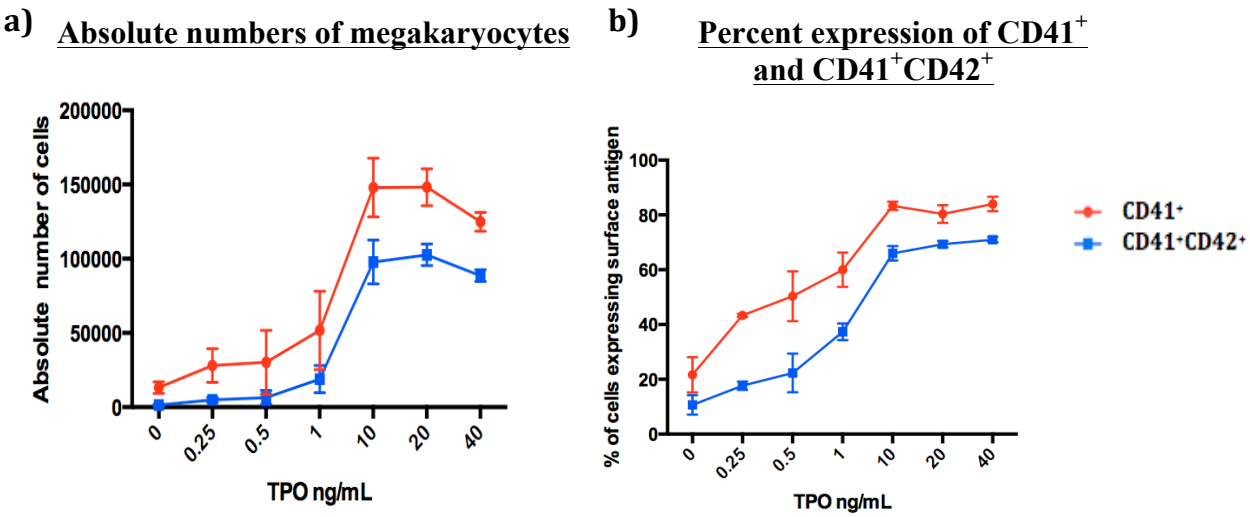
StemSpan expanded $CD34^+$ HSPCs were shown to be TPO dose dependent as well. HSPCs stimulated with 10 ng/mL of TPO for 7 days, post expansion, resulted in maximal percent of cells that were $CD41^+$ and $CD41^+CD42^+$ (Figure 6). Cultures were 71 ± 9.9% $CD41^+$ and 78.1 ± 5.9% $CD41^+CD42^+$. HSPCs from donor B stimulated with 10 ng/mL of TPO resulted in maximal $CD41^+$ and $CD41^+CD42^+$ megakaryocyte numbers while HSPCs from donor C showed maximal megakaryocyte numbers with 20 ng/mL of TPO. Therefore, StemSpan expanded $CD34^+$ HSPCs can be stimulated with 20 ng/mL of TPO to culture the maximal number of mature megakaryocytes.

These results showed that both expansion systems have the ability to expand peripheral blood derived $CD34^+$ HSPCs. $CD34^+$ HSPCs expanded from either system can be stimulated with 20 ng/mL of TPO to produce large numbers of mature megakaryocytes. The larger percentage of mature megakaryocytes in the SR1 cultures may be a direct result of supplementing the expanded $CD34^+$ HSPCs with TPO twice during culturing; StemSpan expanded $CD34^+$ HSPCs were supplemented with TPO on a single occurrence. StemSpan was superior to the SR1 system for $CD34^+$ HSPC expansion, required a smaller time period for expansion and had the added benefit of being commercially available. These qualities made StemSpan a good candidate for peripheral blood derived $CD34^+$ cell expansion and would increase feasibility of the megakaryocyte/CTL co-culture assay. Therefore, StemSpan was used to expand healthy donor $CD34^+$ HSPCs for testing in the CTL assays (Table IV).

Table II: Expansion of peripheral blood derived $CD34^+$ HSPCs from healthy controls the StemSpan expansion system and culturing of megakaryocytes. $CD34^+$ HSPCs (n=4) were expanded for 4 days using StemSpan. The average CD34 purity on day 0 was 92.2 ± 5.2%. Following expansion, the average fold increase of $CD34^+$ cell numbers was 8.73 ± 2.9 and average CD34 purity was 94.6 ± 3.9%. Expanded cells were further cultured with 20 ng/mL of TPO for 7 days to assess megakaryocyte growth. On day 11, the average percent of cells that were $CD41^+$ was 71.1 ± 9.9% and cells that were $CD41^+CD42^+$ was 78.1 ± 5.9%.

	Day 0		Day 4		Day 11	
Donor	**%CD34**	**Yield**	**Expansion (fold)**	**%CD34**	**% of total cells**	
					$CD41^+$	**$CD41^+CD42^+$**
A	98.0	100,000	6.7	93.0	80.0	80.0
B	93.0	160,000	12.9	97.0	70.0	70.0
C	85.3	260,000	8.23	90.0	76.9	78.5
D	92.4	320,000	7.08	98.5	57.6	83.9

Donor B



Donor C

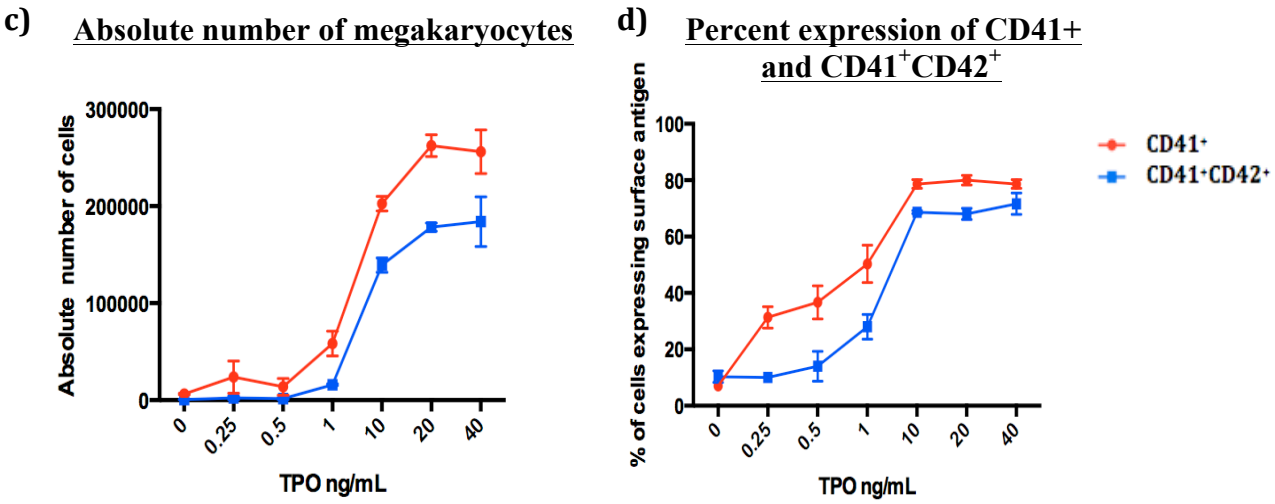


Figure 6 – Determining the optimal TPO dose to culture megakaryocytes from StemSpan expanded CD34$^+$ HSPCs. Healthy donor HSPCs (n=2; donor B and C) were stimulated with varying concentrations of TPO (0, 0.25, 0.50, 1, 10, 20 and 40 ng/mL) after expansion with StemSpan. HSPCs were cultured for 7 days and were assessed for **a)** and **c)** absolute numbers of megakaryocytes and **b)** and **d)** the percent of total cells that were CD41$^+$ (red) and cells that were CD41$^+$CD42$^+$ (blue). The average percent of cells that were CD41$^+$ in the two donors was 71 ± 9.9% and 78.1 ± 5.9% of cells were CD41$^+$CD42$^+$ with 10 ng/mL of TPO stimulation. Maximal absolute numbers of megakaryocytes were achieved **a)** in donor B with 10 ng/mL of TPO stimulation and **c)** in donor C with 20 ng/mL of TPO stimulation.

Table III: Comparing $CD34^+$ expansion systems for peripheral blood

Expansion system	Components	Expansion period	$CD34^+$ fold expansion	TPO dose dependent	Commercially available
StemSpan	SCF, IL-3, IL-6, Flt3L, TPO	4 days	8.72 ± 2.9	Yes	Yes
SR1 (Boitano et al, 2010)	100 ng/mL of SCF, Flt3L, IL-6, TPO, 1 μM SR1	6 days	4	Yes	No

Table IV: Expansion of peripheral blood (90 mL) derived CD34$^+$ HSPCs of healthy controls using StemSpan. Consented healthy donors (n=10) were assessed for expansion using StemSpan. CD34$^+$ cell yield and purity (%) were assessed on day 0 (day of isolation) and on day 4 (after expansion with StemSpan). Donors highlighted in grey were used in the megakaryocyte apoptosis studies. The * symbol indicates that donor CD34$^+$ cells have previously been expanded using StemSpan.

Donor	Day 0		Day 4	
	%CD34	Yield	Expansion (fold)	%CD34
A	85.3	258,700	8.23	90.0
B	92.4	318,500	7.08	98.5
C	87.8	29,000	11.40	98.1
D	98.9	127,000	9.62	96.8
E	91.1	74,200	12.42	97.0
F	57.5	62,600	5.56	95.7
G	75.6	74,200	3.72	97.3
A	93.3	199,500	6.70	94.8
B	90.1	403,800	3.53	98.0
H	61.3	33,300	10.47	96.2
I	91.1	40,100	2	-
J	-	21,000	-	-
G*	85.2	39,800	4.9	-
E	88.4	61,000	8.0	94.9
A*	78.4	113,600	7.9	96.5

4.2 MEASURING ANTI-CD3 AND PEPTIDE INDUCED T CELL STIMULATION IN THE IFN-γ ELISPOT

Blood samples were collected from healthy donors and an IFN-γ ELISpot assay was carried out with freshly prepared PBMCs. Cells were incubated with no peptide, as a

negative control, anti-CD3, a positive control for T cell stimulation and viral CEF peptides, a positive control for peptide induced $CD8^+$ T cell stimulation. T cell stimulation in healthy donor PBMCs (A, B and C) is presented in Figure 7. T cell stimulation was defined by the release of IFN-γ and quantitatively measured by the number of spots observed in the ELISpot. Despite variability in the level of IFN-γ secretion among healthy donors, similar patterns were observed. The mean number of spots in each condition, among all three donor ELISpots was determined. A few spots were counted with no peptide (mean number of spots = 8 ± 7), a moderate number of spots with CEF peptides (mean number of spots = 90 ± 60) and the greatest response seen with anti-CD3 (mean number of spots = 637 ± 196). The average T cell response in "anti-CD3" condition was significantly different from the "no peptide" condition ($p < 0.0001$) and the "CEF peptide" condition ($p < 0.0001$). The average T cell response in the "CEF peptide" condition was not significantly different from the "no peptide" condition ($p = 0.3298$). T cell stimulation that resulted from PBMC incubation with CEF peptides was on average 14% of that observed during anti-CD3 incubation.

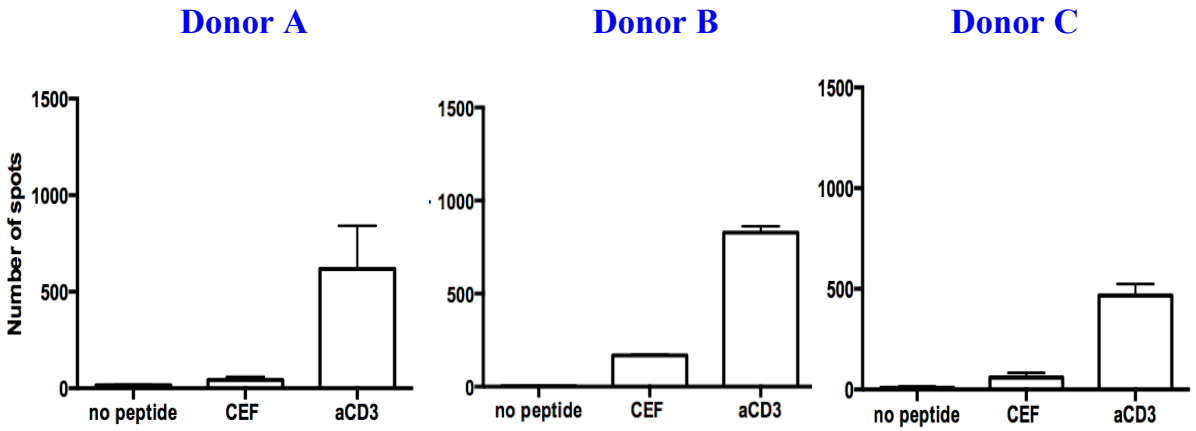


Figure 7 – IFN-γ ELISpot for healthy donor PBMCs. T cell responses were measured by IFN-γ secretion (# of spots) in healthy donors (n=3; donors A, B and C). PBMCs were incubated with no peptide, CEF viral peptides (CEF) at 1 μg/mL of each peptide and anti-CD3 (aCD3) at 1 μg/mL. The mean number of spots across all three donors was deetermined to be: 8 ± 7 in "no peptide", 90 ± 60 in "CEF" and 637 ± 196 in "aCD3." The average T cell response during PBMC incubation with no peptide is significantly different than that observed during anti-CD3 incubation ($p < 0.0001$). The average T cell response in the "CEF" condition was also significantly different from the average response in the "aCD3" condition ($p < 0.0001$). The differences in T cell response between the "no peptide" and "CEF" condition was not significant ($p = 0.3298$). Performed in triplicates. Mean ± SD.

4.3 MEASURING ANTI-CD3 AND PEPTIDE INDUCED CD8⁺ T CELL STIMULATION IN THE IFN-γ ELISPOT

T cell activation induced by CEF peptide presentation was measured in healthy donor (D and E) PBMCs, PBMCs depleted of $CD8^+$ T cells and $CD8^+$ T cells to confirm the specificity of CEF peptides for $CD8^+$ T cell stimulation (Figure 8). Despite variability in the level of IFN-γ secretion in healthy donor PBMC cultures, similar patterns were observed and means T cell responses across the two donors was determined. A few spots were counted with no peptide (mean number of spots = 37 ± 10), a moderate number of spots with CEF peptides (mean number of spots = 307 ± 286) and the greatest response

seen with anti-CD3 (mean number of spots = 869 ± 500). The average T cell response in "anti-CD3" condition was significantly different from the "no peptide" condition (p = 0.0002) and the "CEF peptide" condition (p = 0.0056). The average T cell response in the "CEF peptide" condition was not significantly different from the "no peptide" condition (p value = 0.2088).

When PBMCs were depleted of the $CD8^+$ T cell subset, a few spots were counted in the "no peptide" (mean number of spots = 27 ± 23) and "CEF peptide" (mean number of spots = 13 ± 16) conditions and the greatest number of spots were counted in the "anti-CD3" condition (mean number of spots = 390 ± 273). The depletion of the $CD8^+$ T cell subset resulted in significant loss of T cell response (IFN-γ secretion) during CEF peptide incubation. Compare 307 ± 286 spots, before CTL depletion and 13 ± 16 spots, after CTL depletion (p = 0.0103). This data suggested that T cell stimulation in response to CEF viral peptides was $CD8^+$ T cell specific. The average T cell response to anti-CD3 was significantly reduced after CTL depletion [compare the number of spots before depletion: 869 ± 500 and after depletion: 390 ± 273 (p = 0.0260)] confirming that anti-CD3 stimulates T cells non-specifically and that a majority of IFN-γ secretion from PBMCs is not from $CD8^+$ T cells.

$CD8^+$ T cells that were selected in isolation were also assessed in the ELISpot. Similar patterns of secretion were seen among all healthy donor $CD8^+$ T cells, with a few spots counted with no peptide (mean number of spots = 4), a moderate number of spots with anti-CD3 (mean number of spots = 89 ± 49) and the greatest response seen with CEF peptides (mean number of spots = 143 ± 54). The CTL response observed during $CD8^+$ T

cell incubation with CEF peptides and anti-CD3 was not significantly different ($p = 0.1042$). Additionally, we observed that the combined IFN-γ release (number of spots) in the "anti-CD3" condition obtained from both the $CD8^+$ T cell and the PBMC depleted of $CD8^+$ T cells populations did not have an additive effect adding up to the IFN-γ release in the PBMC population.

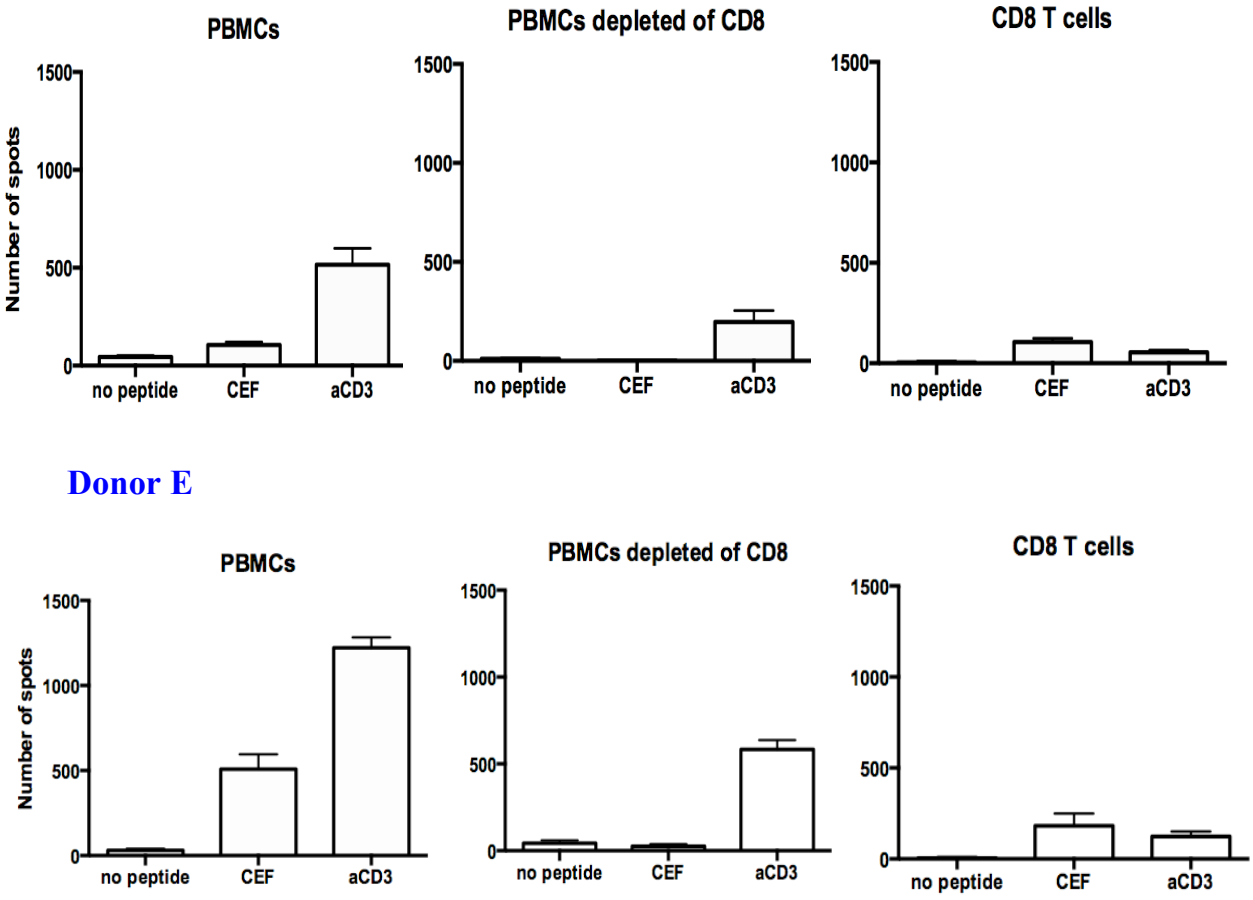


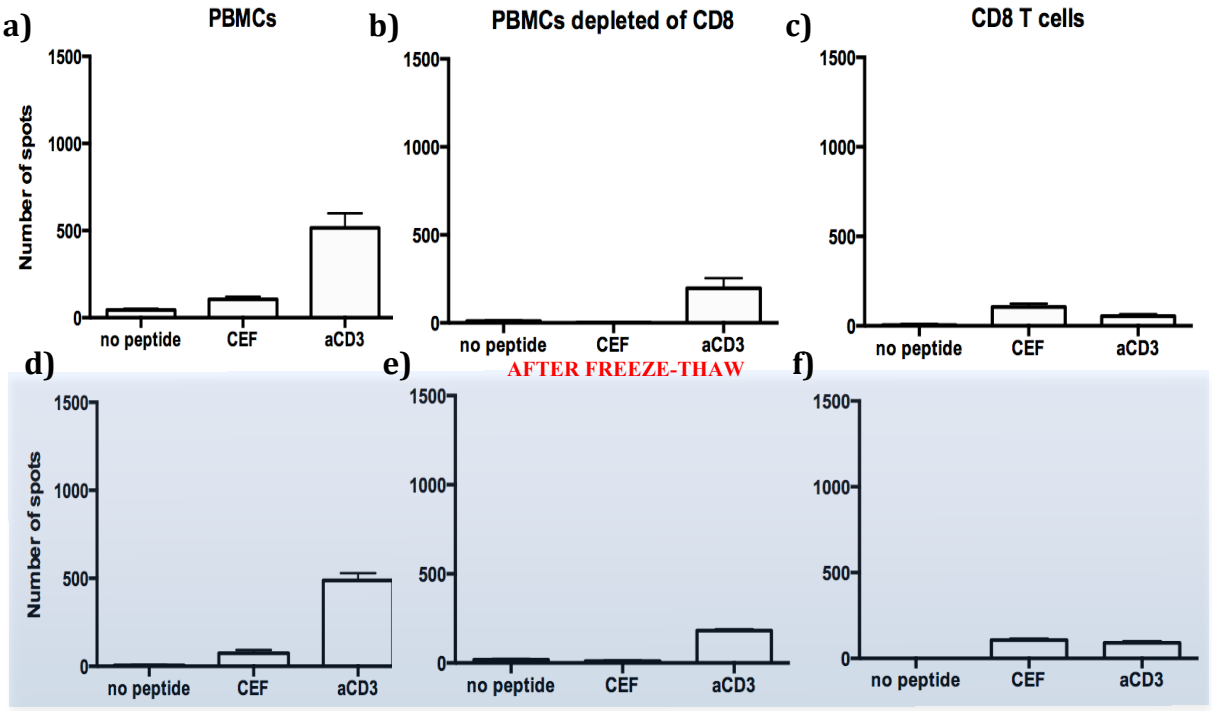
Figure 8 – IFN-γ ELISpot for healthy donor PBMCs, PBMCs depleted of $CD8^+$ T cells and $CD8^+$ T cells. T cell responses were measured by IFN-γ secretion (# of spots) in healthy donors (n=2; donors D and E). PBMCs, PBMCs depleted of $CD8^+$ T cells and $CD8^+$ T cells were incubated with no peptide, CEF viral peptides (CEF) at 1 μg/mL of each peptide and anti-CD3 (aCD3) at 1 μg/mL. The average T cell response in the "CEF" condition during co-culture with PBMCs and PBMCs depleted of $CD8^+$ T cells was 307 ± 286 spots and 13 ± 16 spots respectively. This reduction in T cell response was significant ($p = 0.0103$), suggesting that T cell stimulation in response to CEF peptides is $CD8^+$ T cell specific. The average T cell response in the "aCD3" condition during co-culture with PBMCs and PBMCs depleted of $CD8^+$ T cells was 869 ± 500 spots and 390 ± 273 spots respectively. This reduction in T cell response to anti-CD3 was significant ($p = 0.0260$) confirming that anti-CD3 stimulates T cells non-specifically. Performed in triplicates. Mean ± SD.

4.4 IMPACT OF FREEZE-THAW ON CD8$^+$ T CELL RESPONSES IN THE IFN-γ ELISPOT

To increase the feasibility of this study and avoid multiple donor blood draws it was necessary to assess whether CTLs could be prepared on the same day as HSPCs and be cryopreserved while HPSCs were cultured into megakaryocytes. Healthy donor D and E PBMCs, PBMCs depleted of CD8$^+$ T cells and CD8$^+$ T cells were cultured with no peptide, CEF peptides or anti-CD3 in the IFN-γ ELISpot after cryopreservation and thawing. T cell responses in the ELISpot were measured before and after freezing using donor cells from a single blood sample (Figure 9). Donor D showed comparable responses before and after freezing in all cell populations except for 2 conditions: PBMCs depleted of CD8$^+$ T cells incubated with CEF peptides ($p = 0.0088$) and CD8$^+$ T cells incubated with anti-CD3 ($p = 0.0122$). Donor E showed significantly decreased reactivity in the ELISpot after freeze-thaw in the PBMC and PBMC depleted groups. In the CD8$^+$ T cell population, T cell stimulation was comparable before and after freeze-thaw for both donors, in all conditions, except for the "anti-CD3" condition for donor D [compare the number of spots observed with fresh and cryopreserved cell populations, donor D – fresh: CEF: 105 ± 19, anti-CD3: 54 ± 11 (Figure 9c); cryopreserved, CEF: 107 ± 8 ($p = 0.8540$), anti-CD3: 91 ± 10 ($p = 0.0122$) (Figure 9f); E – fresh: CEF: 182 ± 67, anti-CD3: 124 ± 27 (Figure 9i); cryopreserved, CEF: 189 ± 22 ($p = 0.8540$), anti-CD3: 59 ± 10 ($p = 0.0529$) (Figure 9l)]. A significant difference in CTL response was determined for donor D, CD8$^+$ T cells, in the "anti-CD3" condition, but freezing did not compromise CTL response, where the number of spots observed after freezing was increased. Therefore, using CD8$^+$

T cells after freeze-thaw in the autologous megakaryocyte/CTL co-culture assays to investigate CTL pathology in ITP is reasonable.

Donor D



Donor E

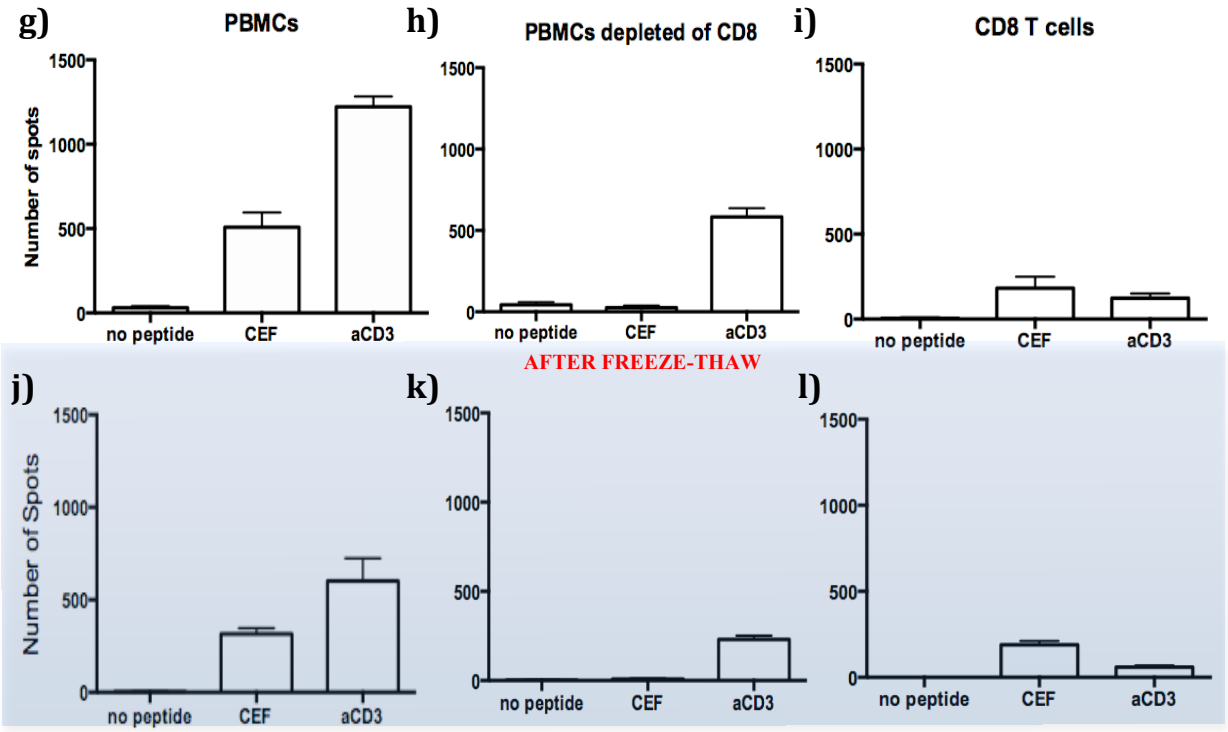


Figure 9 – IFN-γ release during the incubation of healthy donor PBMCs, PBMCs depleted of $CD8^+$ T cells and $CD8^+$ T cells with viral CEF peptides and anti-CD3, before and after freeze-thaw. (n = 2; donor D and E) PBMCs, PBMCs depleted of $CD8^+$ T cells and $CD8^+$ T cells were incubated with no peptide, CEF viral peptides (CEF) at 1 μg/mL of each peptide and anti-CD3 (aCD3) at 1 μg/mL, before and after cryopreservation. Cryopreservation of cell populations in donor D showed no significant changes ($p > 0.05$) in T cell response in the IFN-γ ELISpot compared to using fresh samples except for 2 conditions: PBMCs depleted of $CD8^+$ T cells incubated with CEF peptides [CTL responses using fresh versus cryopreserved cells, number of spots – fresh **b)** 2 ± 2, cryopreserved **e)** 16 ± 5 ($p = 0.0088$)] and $CD8^+$ T cells incubated with anti-CD3 [number of spots – fresh **c)** 54 ± 11, cryopreserved **f)** 91 ± 10 ($p = 0.0122$). Donor E cell populations showed significantly decreased ($p < 0.05$) reactivity in the ELISpot after freeze-thaw in **g)** and **j)** the PBMC condition and **h)** and **k)** the PBMC depleted of $CD8^+$ T cells condition. T cell stimulation in the $CD8^+$ T cell population was comparable before and after freeze-thaw for both donors in all conditions [number of spots, D – fresh **c)** CEF: 105 ± 19, anti-CD3: 54 ± 11; cryopreserved **f)** CEF: 107 ± 8 ($p = 0.8540$), anti-CD3: 91 ± 10 ($p = 0.0122$); E – fresh **i)** CEF: 182 ± 67, anti-CD3: 124 ± 27; cryopreserved **l)** CEF: 189 ± 22 ($p = 0.8540$), anti-CD3: 59 ± 10 ($p = 0.0529$)]. Note: an increased numbers of spots were observed in the "anti-CD3" condition for donor D after freeze-thaw. Performed in triplicates. Mean ± SD.

4.5 $CD8^+$ T CELL RESPONSES IN THE PRESENCE OF AUTOLOGOUS PLATELETS IN THE IFN-γ ELISPOT

There is currently no literature available on the ability of megakaryocytes to present antigen in the context of MHC class I. Contrarily, platelets have been shown to express T cell co-stimulatory molecules, process and present antigen to $CD8^+$ T cells via MHC class I, leading to T cell activation (Chapman et al, 2012). For our study, megakaryocytes will be incubated with viral CEF peptides and subsequently co-cultured with CTLs to induce memory CTL activation due to specific antigen and to model peptide presentation by megakaryocytes to CTLs. Therefore, prior to conducting megakaryocyte experiments, it was beneficial to confirm findings in the literature regarding T cell stimulation due to platelets presenting peptide antigen using the IFN-γ ELISpot. It was

speculated that this experiment would provide good insight as to whether megakaryocytes could present specific antigen in the context of MHC class I leading to CTL stimulation, since platelets share many of the same protein receptors as their precursor cells.

Healthy donor $CD8^+$ T cells (A, B and C) were tested in an IFN-γ ELISpot with autologous platelets. Platelets were co-cultured with CTLs and incubated with no peptide, viral CEF peptides and anti-CD3 (Figure 10). The mean number of spots across all three donors was determined. Platelets were incubated with CEF peptides as a negative control and resulted in a very low background (mean number of spots: 1 ± 1). As a second control, T cells were incubated with CEF peptides alone and resulted in a large response (mean number of spots = 48 ± 38). Similar patterns in IFN-γ secretion were exhibited with donor platelet/CTL co-cultures, with a few spots counted with no peptide (mean number of spots: 4 ± 6), a moderate number of spots with CEF peptides (mean number of spots = 31 ± 9) and the greatest response was seen with anti-CD3 (mean number of spots = 50 ± 28). There was no significant difference observed when T cells were incubated with CEF peptides compared to when T cells and platelets were incubated with CEF peptides ($p = 0.2417$).

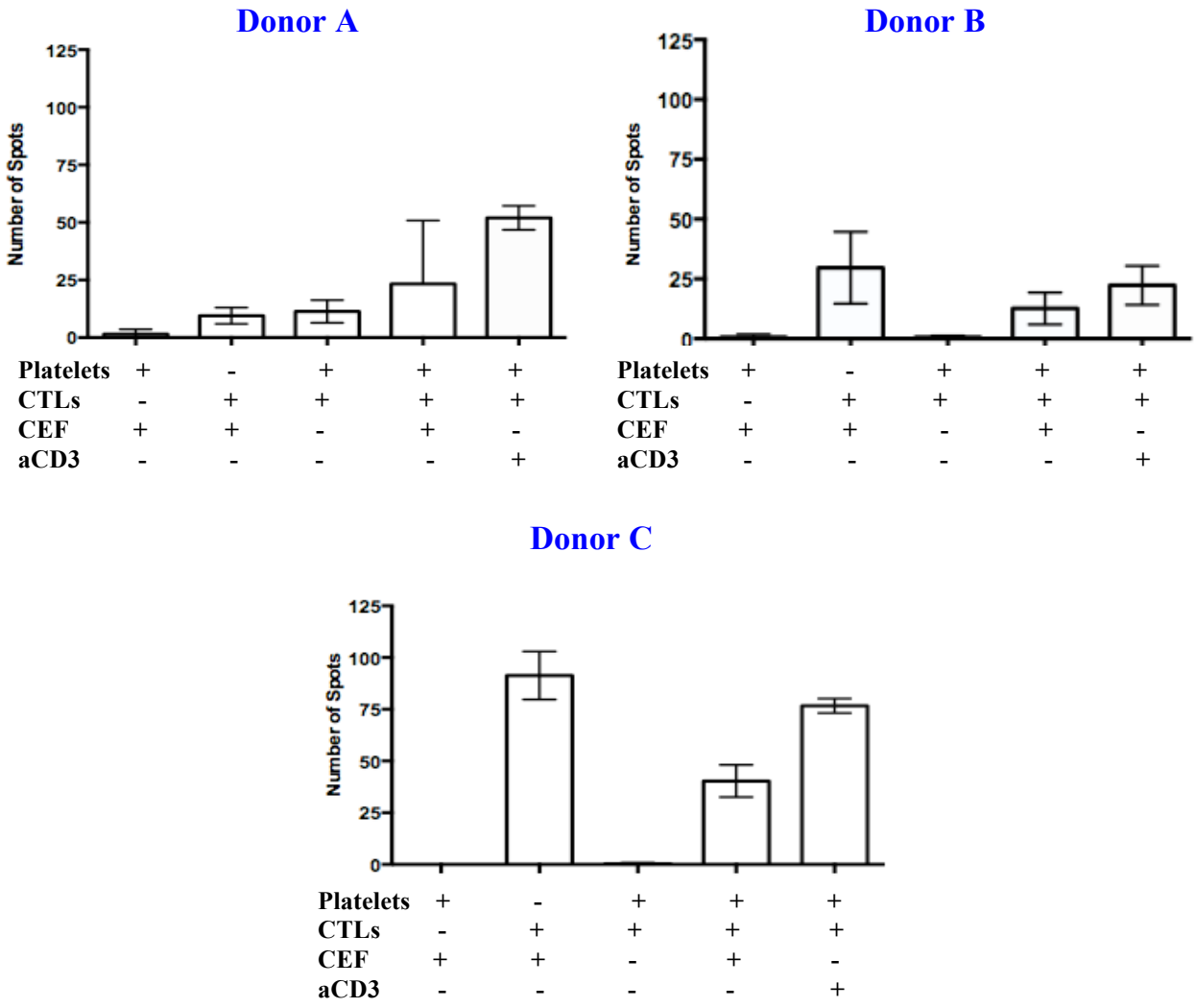


Figure 10 – Platelets as peptide presenting cells in the context of MHC class I: IFN-γ ELISpot for healthy donor platelet/CTL co-cultures. Platelets were incubated with autologous CTLs and then further incubated with no peptide, CEF viral peptides (CEF) or anti-CD3 (aCD3) (n = 3; donors A, B and C). Platelets were incubated with CEF peptides 1 hour prior to co-culturing with CTLs. Anti-CD3 was incubated with CTLs prior to co-culturing with autologous platelets. The controls included platelets incubated with CEF peptides, CTLs incubated with CEF peptides and platelets co-cultured with CTLs and no peptide. The mean number of spots across all three donors was deetermined to be: 1 ± 1 in "platelets + CEF", 48 ± 38 in "CTLs + CEF", 4 ± 6 in "no peptide", 31 ± 9 in "CEF" and 50 ± 28 in "aCD3." The "CEF" condition, in which platelets were incubated with CEF peptides and CTLs did not result in significantly different IFN-γ secretion compared to when CTLs were incubated with CEF peptides alone (p=0.2417). Performed in triplicates. Mean ± SD.

4.6 THE IMPACT OF HEALTHY DONOR CD8$^+$ T CELLS ON MATURE MEGAKARYOCYTE APOPTOSIS

A single publication has attributed platelet underproduction in ITP to be the result of reduced megakaryocyte apoptosis when CTLs are present during megakaryopoiesis (Li et al, 2007). We hypothesize that platelet underproduction may be due to mature megakaryocyte apoptosis. To test this hypothesis *in-vitro*, we developed a megakaryocyte apoptosis assay using megakaryocytes and autologous CTLs from healthy controls. High purity PB derived CD34$^+$ HSPCs (> 85%) were prepared from healthy donor A, expanded (Table IV) and used to culture mature megakaryocytes (78.5% CD41$^+$CD42$^+$). The impact of CD8$^+$ T cell (CTL) incubation on mature megakaryocyte apoptosis was assessed by comparing annexin-V labeling in co-cultures of megakaryocytes (CD41$^+$ cells) with PBMCs, PBMCs depleted of CD8$^+$ T cells and CD8$^+$ T cells. Co-cultures were incubated with or without viral CEF peptides (1 μg/mL of each peptide). Annexin-V labeled 37.9 ± 1.3% of megakaryocytes (CD41$^+$) cultured alone. Megakaryocytes in all other co-culture conditions were labeled with a lower percentage of annexin-V compared to CD41$^+$ cells cultured alone (Table V). Visually, light microscopy revealed that PBMC and PBMC depleted of CD8$^+$ T cell co-cultures exhibited high levels of clumping where rosette like structures of mononuclear cells had formed around megakaryocytes compared to the other culture conditions.

Table V: Measuring healthy donor megakaryocyte apoptosis. Healthy donor (n=1; donor A) megakaryocyte ($CD41^+$ cells) apoptosis was measured on day 12 (7 days post expansion) cells after incubation with autologous PBMCs (1:160), PBMCs depleted of $CD8^+$ T cells (1:140) and $CD8^+$ T cells (1:20), with or without CEF peptides (CEF) overnight. Baseline megakaryocyte apoptosis was determined to be 37.9 ± 1.3%. A positive control for megakaryocyte apoptosis was not included. All co-culture conditions showed significantly reduced megakaryocyte annexin-V labeling ($p < 0.05$). This was speculated to be the consequence of increased cell clumping. Media discoloration was noted in the PBMC and $CD8^+$ T cell (CTL) depleted subset co-cultures. Performed in triplicates. Mean ± SD.

Co-culture groups	Average %$CD41^+$ cells labeled with Annexin-V
CD41	37.9 ± 1.3
CD41 + PBMCs	9.58 ± 2.0
CD41 + CEF + PBMCs	10.9
CD41 + PBMCs depleted of CTLs	9.66 ± 2.6
CD41 + CEF + PBMCs depleted of CTLs	9.18
CD41 + CTLs	27.7 ± 1.2
CD41 + CEF + CTLs	30.3 ± 0.7

The impact of $CD8^+$ T cell (CTL) incubation on megakaryocyte apoptosis was assessed a second time by comparing annexin-V labeling during megakaryocyte co-culture with CTLs only. High purity PB derived $CD34^+$ HSPCs (92.4%) were prepared from healthy donor B, expanded (Table III) and used to culture mature megakaryocytes (83.9% $CD41^+CD42^+$). Megakaryocytes were cultured with CTLs at a ratio of 1:10 and 1:20 and incubated with no peptide, CEF peptides (1 μg/mL of each peptide) and anti-CD3 (5 μg/mL). In this experiment, 44.0 ± 0.6% of megakaryocytes ($CD41^+$ cells) cultured alone were labeled with annexin-V compared to 92.9 ± 5.6% of the calcium ionophore (positive control) treated megakaryocytes (Table VI). Megakaryocytes co-cultured with CTLs at a 1:10 ratio were labeled with significantly less annexin-V compared to megakaryocytes cultured alone (35.0 ± 0.9%). There was no significant

difference in the annexin-V labeling when megakaryocytes were cultured with CTLs (1:10 ratio) and anti-CD3 or CEF peptides. At the 1:20 ratio, the presence of CTLs did not impact the level of annexin-V labeling on megakaryocytes. When the co-culture was incubated with anti-CD3, megakaryocytes were labeled with significantly increased levels of annexin-V (61.8 ± 2.8%, $p < 0.0001$) compared to megakaryocytes cultured alone or with CTLs.

Table VI: Measuring healthy donor megakaryocyte apoptosis in $CD41^+/CD8^+$ T cell co-cultures. Healthy donor (n=1; donor B) megakaryocyte apoptosis was measured by determining the percent of $CD41^+$ cells labeled with annexin-V ($CD41^+$annexin-V^+) on day 12 (7 days post expansion). Baseline megakaryocyte apoptosis was determined to be 44.0 ± 0.4%. Maximal megakaryocyte apoptosis induced by calcium ionophore was determined to be 92.9 ± 5.6% and was significantly different from baseline ($p < 0.001$). Annexin-V labeling was significantly increased on megakaryocytes in the presence of anti-CD3 stimulated CTLs at the 1:20 ratio of target to effector cells ($p < 0.001$). No increase in megakaryocyte annexin-V labeling was observed in the presence of CEF peptide stimulated CTLs in this donor. The co-culture of CTLs with $CD41^+$ cells resulted in significantly reduced annexin-V labeling at the 1:10 ratio ($p = 0.0201$) but did not impact annexin-V labeling at the 1:20 ratio. While annexin-V labeling on megakaryocytes was increased in the presence of anti-CD3 stimulated CTLs (1:20), it cannot confirm megakaryocyte apoptosis. Performed in triplicates. Mean ± SD. The * symbol indicates annexin-V labeling that is significantly different from the "CD41" condition.

Culture conditions	**Average %$CD41^+$ cells labeled with Annexin-V**
CD41	44.0 ± 0.4
CD41 + calcium Ionophore	92.9 ± 5.6*
CD41 + CTLs (1:10)	35.0 ± 0.9 *
CD41 + CEF + CTLs	47.1 ± 3.6
CD41 + CTLs + aCD3	48.1 ± 3.3
CD41 + CTLs (1:20)	43.6 ± 3.4
CD41 + CEF + CTLs	45.2 ± 1.7
CD41 + CTLs + aCD3	61.8 ± 2.8*

4.7 THE IMPACT OF HEALTHY DONOR CD8$^+$ T CELLS ON MEGAKARYOCYTE GROWTH

4.7.1 Optimizing the Peripheral Blood Megakaryopoiesis Assay

The megakaryopoiesis assay was optimized for the number of HSPCs initially plated and the number of days required for culturing a sufficient number of megakaryocytes for analysis using 10 ng/mL of TPO. Megakaryocyte cultures were initiated with 20,000 HSPCs (donor C) for 6 days and 10,000 HSPCs (donor G) for 7 days with 10 ng/mL of TPO. HSPC CD34$^+$ purity at time of co-culturing was > 95% for both donors (Table IV). On day 6, donor C HSPC cultures yielded 24,500 CD41$^+$ cells, a 1.2-fold increase in cell count. On day 7, donor G HSPC cultures yielded 21,600 CD41$^+$ cells, a 2.1-fold increase in cell count. These results prompted us to initiate all future megakaryocyte cultures with 10,000 HSPCs stimulated with 10 ng/mL of TPO for 7 days.

4.7.2 Measuring the Impact of CD8$^+$ T Cells on the Quantity of Megakaryocytes

In addition to observing reduced megakaryocyte apoptosis in the presence of CTLs in ITP samples, Li and colleagues observed reduced megakaryocyte ploidy (Li et al, 2007). Our goal was to design a CTL assay to measure inhibition of megakaryocyte growth using healthy donors so that we can apply it to ITP samples and confirm previous preliminary findings in literature. In order to do this, HSPCs were co-cultured with autologous CTLs at a ratio of 1:10 and stimulated with TPO (10 ng/mL) for 7 days. Four healthy donors were tested and repeat testing was carried out for two donors. The number and percentage of megakaryocytes (CD41$^+$) and mature megakaryocytes (CD41$^+$CD42$^+$) were assessed in culture. The presence of CTLs during *in-vitro* health donor

megakaryopoiesis of HSPCs did not impact the total number and percentage of $CD41^+$ cells on day 11 (day 7, post expansion) of co-culturing (Table VII). The presence of CTLs during megakaryopoiesis yielded increased numbers and percentages of mature megakaryocytes on day 11 in three out of four donors (A, E and B) (Table VIII). The presence of CTLs and viral CEF peptides during megakaryopoiesis resulted in variable inhibition of megakaryocyte growth among healthy donors. Megakaryocyte lineage commitment and maturation during CEF peptide and CTL incubation was most greatly inhibited in donor E, moderately inhibited in donor G and least inhibited in donors A and B. Inhibition of megakaryopoiesis was observed in all donors during HSPC/CTL co-culture incubation with anti-CD3 and ranged between 30.1 and 53.9%. Our data shows that inhibition of megakaryopoiesis during CTL co-culture and incubation of either anti-CD3 or CEF peptides can be detected using flow cytometry by measuring $CD41^+$ and $CD41^+CD42^+$ expression.

The mean percent of megakaryocyte lineage commitment ($CD41^+$) and maturity ($CD41^+CD42^+$) among all healthy donors tested for each condition in the megakaryocyte growth inhibition assay was evaluated. Healthy donor HSPCs expressed an average of 69.7 ± 9.7% CD41 after stimulation with TPO for 7 days. The presence of CTLs during megakaryopoiesis did not significantly impact average CD41 expression (71.3 ± 12.8%) compared to HSPCs cultured alone. Despite donor variability, the presence of CTLs and anti-CD3 during healthy donor megakaryopoiesis significantly decreased megakaryocyte lineage commitment, with donors expressing 32.2 ± 11.9% ($p = 0.0255$) CD41 on average (Figure 11a). The presence of CTLs and CEF peptide treated healthy donor, HSPCs

undergoing megakaryopoiesis also resulted in significantly reduced megakaryocyte lineage commitment (40.0 ± 28.7%, p = 0.0306) on average, but the range of inhibition was greater than that seen in the "anti-CD3" condition. Inhibition of megakaryocyte maturity (% of cells that are $CD41^+CD42^+$) was also observed in the presence of CEF peptide and anti-CD3 stimulated CTLs, but these results were not significantly different than megakaryocyte maturity observed during megakaryopoiesis of HSPCs cultured with no CTLs (Figure 11b).

4.7.3 Reproducibility of the Megakaryocyte Growth Inhibition Assay

Repeat testing was carried out for healthy donors G and A, two months following initial testing, with a fresh 90 mL blood sample. The incubation of CEF peptides during HSPC/CTL co-culture (donor G) resulted in a 46% decrease in $CD41^+$ cells compared to HSCPs cultured alone. Upon repeat testing (donor G*), 71.90 ± 4.0% of cells were $CD41^+$ in the "HSPCs alone" condition compared to 28 ± 5.2% in the "HSPCs + CEF + CTLs" condition. Therefore, the incubation of HSPCs with CEF peptides during CTL co-culture resulted in a 44% decrease in $CD41^+$ cells. For donor A, the incubation of CEF peptides in the HSPC/CTL co-culture resulted in a 19% decrease in CD41 expression compared to HSCPs cultured alone (p = 0.043). Upon repeat testing (donor A*), 74.0 ± 6.8% of cells were $CD41^+$ in the "HSPCs alone" condition compared to 72.2 ± 6.1% in the "HSPCs + CEF + CTLs" condition. No significant inhibition of megakaryocyte growth was observed with donor A* (p = 0.9721). While donor G showed moderate inhibition of megakaryocyte growth in the presence of CEF peptides and CTLs, donor A showed minimal inhibition. The level of inhibition of megakaryocyte growth in the

presence of CTLs and CEF peptides were similar overtime within individual healthy donors. This data showed that CEF peptides can be used as an internal control for assay reproducibility.

4.7.4 Limitations of the Megakaryocyte Growth Inhibition Assay

In this study, peripheral blood (90 mL) derived $CD34^+$ cell yield (n=10) ranged from 21,000 to 403,700 on day 0, while $CD34^+$ cell expansion with StemSpan ranged from a 3.53-fold to 12.42-fold increase. High $CD34^+$ cell yield and purity on day 0 was not always associated with high fold increase of $CD34^+$ cell numbers during the 4-day expansion period. The limiting factor for conducting the HSPC/CTL co-culture assay was a low $CD34^+$ cell yield after expansion. On day 4, the minimum number of $CD34^+$ cells required for co-culturing was 3×10^5 $CD34^+$ cells. Donor derived $CD34^+$ cultures from healthy donors I and J did not meet the minimum $CD34^+$ cell yield to initiate the HSPC/CTL co-culture assay (Table III). $CD8^+$ T cell frequencies in healthy donors ranged between 7.2×10^6 and 4.2×10^7 cells. After freeze-thaw, the recovery rate for $CD8^+$ T cell numbers was between 60-70% and cell viability was > 90%. The minimum number of $CD8^+$ T cells required for the assay was 1.2×10^6. These cells were easily harvested and always present in excess.

Early HSPC/CTL co-culture data was analyzed via flow cytometry using CD41 and CD42 antibody bound fluorophores. This method of analysis prevented the direct comparison of percent of cells that were $CD41^+$ and $CD41^+CD42^+$ between the "HSPCs alone" and the HSPC/CTL co-culture conditions. Upon repeat testing for donors A and G and for the initial testing for donor E, a CD8 antibody bound fluorophore was included to

exclude CD8 positive events during flow cytometry analysis. By excluding CD8 positive events we achieved increased accuracy in measuring CD41 and CD42 positive events and it became possible to compare percent of $CD41^+$ cells and $CD41^+CD42^+$ cells between all culture conditions. The location of CD8 positive events on the forward vs. side scatter profile became known after $CD8^+$ event exclusion. Based on location, CD8 positive events were removed from previous healthy donor, megakaryocyte growth inhibition data and re-analyzed. Only slight differences were observed in the absolute numbers of cells that were $CD41^+$ and $CD41^+CD42^+$ between the two methods of analysis.

Table VII: Measuring inhibition of megakaryocyte lineage commitment in healthy donors: absolute numbers and percentage of $CD41^+$ cells on day 11. Inhibition of megakaryocyte lineage commitment was measured in healthy controls (n=4; donors G, A, B and E). Two of the four donors were re-tested (G* and A*). Assay conditions were performed in triplicates, and in singlets for donor G due to limited numbers of HSPCs. Donors G, E and A showed significant inhibition in megakaryocyte lineage commitment ($CD41^+$ cells) when TPO stimulated HSPCs were cultured with CEF peptide stimulated CTLs or anti-CD3 stimulated CTLs ($p < 0.05$). Donors A* and B showed no significant inhibition when HSPCs were cultured with stimulated CTLs. Anti-CD3 stimulated CTLs mediated inhibition of megakaryocyte lineage commitment in all donors tested, while CEF peptide stimulated CTLs mediated inhibition of megakaryocyte lineage commitment in select donors. Means ± SD.

$CD41^+$	HSPCs		HSPCs + CEF		HSPCs + aCD3		HSPCs + CTLs		[HSPCs + CEF] + CTLs		HSPCs + CTLs + aCD3	
Donor	**%**	**Absolute count**	**%**	**Absolute count**	**%**	**Absolute count**	**%**	**Absolute count**	**%**	**Absolute count**	**%**	**Absolute count**
G	55.9	24,547	48.7	30,914			47	29,220	9.8	1,502		
G*	71.9 ± 4.0	22,779 ± 3161					77.2 ± 0.7	23,053 ± 6904	28 ± 5.2*	4,753 ± 714	41.8 ± 6.9*	8,130 ± 809
A	82.8 ± 6.8	31,765 ± 3888	76.6 ± 1.3	18,824 ± 2926			77.2 ± 0.6	21,957 ± 2604	63.6 ± 1.3*	21,874 ± 542	36.0 ± 1.7*	8,689 ± 559
A*	74.0 ± 2.2	55,354 ± 2079	78 ± 5.1	59,431 ± 5139	75.8 ± 5.3	47,361 ±10171	75.8 ± 4.7	39,766 ± 1023	72.2 ± 6.1	49,943 ± 7290		
B	60.9 ± 0.1	15,260 ± 38	46.5 ± 7.7*	10,238 ± 1375			68.0 ± 5.0	18,575 ± 1759	59.2 ± 2.5	21,873 ± 1929		
E	72.8 ± 4.2	39,352 ± 2945	74.9 ± 1.6	25,095 ± 2552	87 ± 1.4*	31,484 ± 4972	82.5 ± 2.4*	36,329 ± 8924	6.9 ± 5.2*	3,844 ± 1964	18.9 ± 1.2*	12,060 ± 1645

Table VIII: Measuring inhibition of megakaryocyte maturity in healthy donors: absolute numbers and percentage of $CD41^+CD42^+$ cells on day 11. Inhibition of megakaryocyte maturity was measured in healthy controls (n=4; donors G, A, B and E). Of the donors tested, one donor was re-tested (A*). Donors G and E showed significant inhibition in megakaryocyte maturity ($CD41^+CD42^+$ cells) when TPO stimulated HSPCs were cultured with CEF peptide stimulated CTLs ($p < 0.05$). Donors A and E showed significant inhibition in megakaryocyte maturity when TPO stimulated HSPCs were cultured with anti-CD3 stimulated CTLs ($p < 0.05$). Performed in triplicates. Means ± SD.

$CD41^+$ $CD42^+$	HSPCs		HSPCs + CEF		HSPCs + aCD3		HSPCs + CTLs		HSPCs + CEF + CTLs		HSPCs + CTLs + aCD3	
Donor	**%**	**Absolute count**	**%**	**Absolute count**	**%**	**Absolute count**	**%**	**Absolute count**	**%**	**Absolute count**	**%**	**Absolute count**
G*	61.8 ± 2.1	14,033 ± 1,485					75.4 ± 0.3	17,372 ± 5,141	21.9 ± 0.2*	1,040 ± 166	50.7 ± 8.0	4,157 ± 1049
A	67.5 ± 0.2	21,444 ± 2,669	73.3 ±1.0*	13,783 ± 1,959			81.0 ±0.2*	17,772 ± 2,061	72.9 ± 1.6*	15,942 ± 55	55.5 ±1.0*	4,820 ± 270
A*	70.9 ± 6.9	39,337 ± 4,782	75.6 ± 1.9	44,848 ± 2,771	82.5 ±2.4*	38,923 ± 7,280	83.7 ±0.2*	33,265 ± 941	83.7 ± 2.7*	41,939 ± 7,358		
B	72.7 ± 3.0	11,334 ± 489	70.7 ± 1.6	7,244 ±1,138			90.7 ±2.0*	16,663 ±1,801	89.4 ±2.5*	19,509 ±2,686		
E	75.2 ± 4.6	29,616 ± 3,428	80.1 ± 3.3	20,14±2, 480	80.0 ± 9.6	25,141 ± 4504	88.3 ±0.9*	32,111 ± 8,049	4.04 ± 1.2*	156 ± 80	34.7 ±2.4*	4,190 ± 703

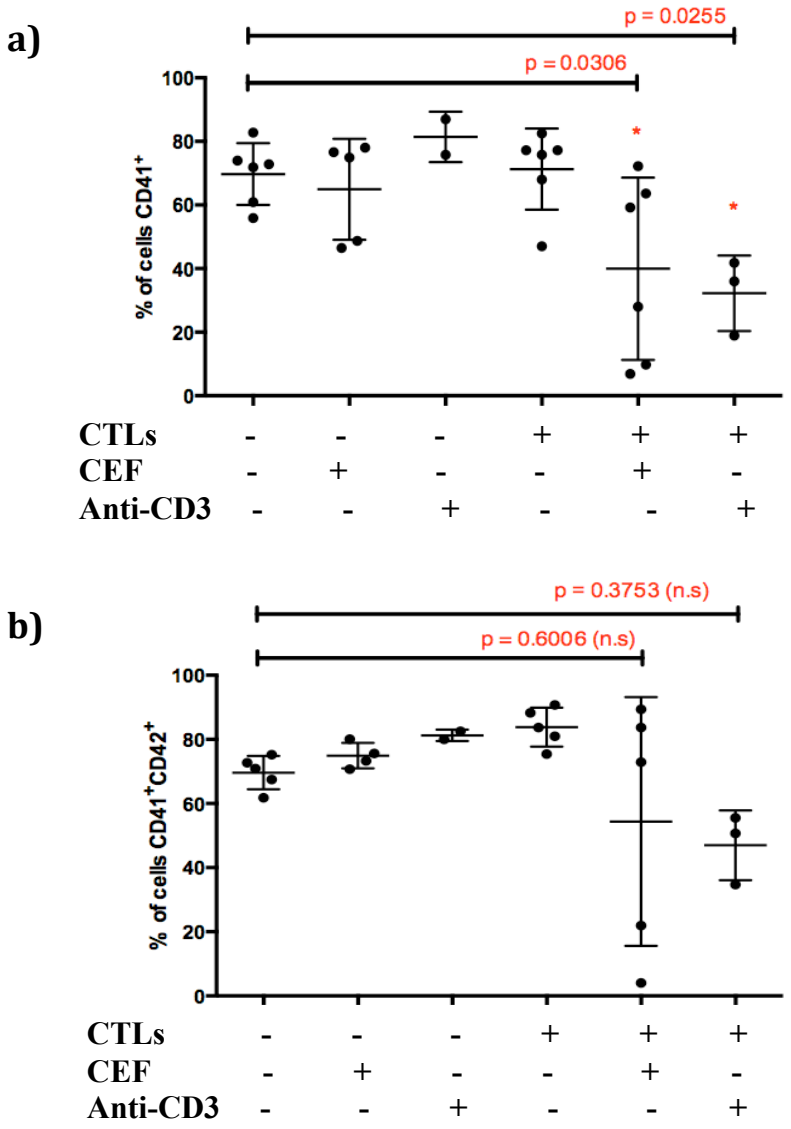


Figure 11 – The mean impact of CTL-mediated inhibition of megakaryocyte growth in healthy controls. Mean percent commitment to the megakaryocyte lineage ($CD41^+$ cells) and megakaryocyte maturity ($CD41^+CD42^+$) among healthy donors (n=4; donors G, A, B and E). Of the donors tested, 2 donors were re-tested and are included in the results. **a)** On average, % of total cells that were $CD41^+$ were significantly decreased when TPO stimulated HSPCs were incubated with CEF peptides and CTLs ($p = 0.0306$) and when they were incubated with CTLs and anti-CD3 ($p = 0.0255$); **b)** On average, % of total cells that were $CD41^+CD42^+$ were not significantly decreased when TPO stimulated HSPCs were incubated with CEF peptides and CTLs ($p = 0.6006$) and when they were incubated with CTLs and anti-CD3 ($p = 0.3753$).

5.0 DISCUSSION

5.1 EXPANDING PERIPHERAL BLOOD DERIVED CD34$^+$ HSPCs WITH STEMSPAN CD34 EXPANSION SUPPLEMENT

It is common practice to obtain hematopoietic stem and progenitor cells (HSPCs), characterized as CD34$^+$, from cord, bone marrow or mobilized peripheral blood and use these cells to produce large numbers of megakaryocytes *in-vitro*. To develop a feasible co-culture assay that can be extended to patient samples, it was necessary to find an alternate source of HSPCs, such as peripheral blood (PB). The main benefit of using PB is that it is more accessible compared to other HSPC sources. PB derived CD34$^+$ cells have also been shown to produce increased numbers of high ploidy megakaryocytes compared to bone marrow or cord blood sources (Mattia et al, 2002). The main limitation is that PB derived HSPCs make up only between 0.03 – 0.09% of total circulating nucleated cells compared to the 1.1% in bone marrow (Korbling & Anderlini, 2001; Sutherland et al, 1994). In our studies, we found that the yield of HSPCs from peripheral blood was much lower in comparison to what is stated in literature. One explanation for reduced yield may be the efficiency of the columns used for CD34 positive selection in the laboratory. To overcome these limitations it was necessary to find a suitable protocol to expand CD34$^+$ cells from PB.

In this study, we found that a combination of SCF, IL-3, IL-6, Flt3L, TPO and other additives present within the StemSpan supplement resulted in an average 7.9-fold increase of PB derived CD34$^+$ cells after 4 days of expansion. StemSpan was found to be more effective in achieving optimal CD34$^+$ cell expansion compared to the SR1

expansion system (SR1, TPO, SCF, 1L-6 and Flt3L). It is speculated that this may in part be explained by the difference in cytokines present within the two expansion systems. While the concentrations for the individual cytokines in the StemSpan supplement were not made publicly available, the main difference between the two expansion systems was that StemSpan consisted of IL-3 and lacked the chemical modulator, SR1. Previous research has shown that when $CD34^+$ cells from PB are cultured with TPO in combination with IL-3, cultures express lower numbers of CD41 expressing cells (van den Oudenrijn et al, 2000). These observations could account for the reduced differentiation to other blood cell lineages (CD41) and increased CD34 cell expansion with StemSpan compared to the SR1 expansion system. Finding an expansion system that resulted in minimal differentiation of HSPCs to other blood cell lineages during the "expansion phase" was important for our studies and for the goal of attaining a large number of megakaryocytes. $CD34^+$ cells that differentiate to other blood lineages (CD41) during the expansion phase cannot undergo further expansion and hence limits expansion potential; small expansion potential limits the number of megakaryocytes that can be cultured during the differentiation phase. In our studies, the SR1 expansion system resulted in increased $CD34^+$ cell differentiation compared to StemSpan and therefore was not the expansion system of choice. During the differentiation phase, although megakaryocytes cultured from SR1 expanded HSPCs exhibited high levels of CD41 and CD42 expression, the numbers of megakaryocytes were less than those grown from StemSpan expanded HSPCs.

SR1 was identified as a small molecule with the greatest potential to expand $CD34^+$ cells within 5-7 days via a high throughput screen (Boitano et al, 2010). Its prior use in expanding HSPCs from mobilized peripheral blood encouraged us to evaluate its use in expanding PB derived $CD34^+$ HSPCs. Furthermore, evidence in the literature shows that SR1 inhibits the actions of the AhR transcription factor which has a role in promoting megakaryopoiesis and more specifically, promoting increased ploidy (Lindsey & Papoutsakis, 2011). In our studies with SR1, it was surprising to find the differentiation of $CD34^+$ cells into CD41 surface antigen bearing cells to not be inhibited – CD41 being a lineage commitment marker for megakaryopoiesis. Poor expansion and increased differentiation observed during SR1 aided HSPC expansion, may in part be attributed to the dose of SR1 used in our experiments. Evidence in the literature shows that SR1 concentrations greater than 1 μM can have an anti-proliferative effect and that a positive impact on expansion in the presence of other cytokines occurs only within a narrow concentration range (Boitano et al, 2010). It is therefore possible that the 1 μM SR1 dose used in our study may not have been optimal for maximal $CD34^+$ cell expansion. Further studies including a SR1 dose experiment would be required to evaluate whether a smaller dose would result in improved expansion of PB derived $CD34^+$ HSPCs.

Through conducting these experiments, we found the StemSpan expansion protocol to fulfill the primary goals of our first objective. StemSpan expanded $CD34^+$ HSPCs from peripheral blood with minimal differentiation to other blood cell lineages, resulted in optimal expansion in 4 days and was commercially available, increasing the

feasibility of its use in assay development. This expansion protocol was therefore used to expand all donor $CD34^+$ HSPCs in this study.

5.2 ACHIEVING A PHYSIOLOGICALLY RELEVANT LEVEL OF $CD8^+$ T CELL STIMULATION TO STUDY AUTOIMMUNE DISEASE

The ELISpot was used to measure CTL stimulation resulting in IFN-γ secretion in response to two positive controls: anti-CD3 and the viral CEF peptide pool. While anti-CD3 induces polyclonal stimulation of T cells, CEF peptides induce the stimulation of a small frequency of antigen specific CTLs. While both methods result in TCR stimulation, studies have shown that the cytokine profile of T cells stimulated with these different agents is not identical (Reinherz et al, 1982). Nikolova and colleagues showed that CTLs stimulated with CEF peptides secreted less IL-2 in the presence of T regulatory cells compared to those stimulated with anti-CD3 (Nikolova et al, 2009). Furthermore, CTL proliferation in the presence of CEF peptides was lower compared to anti-CD3 (Nikolova et al, 2009). In order to model the level of stimulation of autoreactive, antigen specific CTLs that would result in a realistic level of CTL induced inhibition of megakaryocyte growth, we used CEF peptides to stimulate antigen specific CTLs in our studies. Additionally, this study would give us the opportunity to assess the ability of flow cytometry to detect megakaryocyte apoptosis and growth inhibition due to low frequency antigen specific CTL stimulation.

Most studies that use CEF peptides as a positive control for CTL stimulation incubate PBMCs with CEF peptides. There is currently no evidence in the literature for CEF peptide incubation with megakaryocytes and/or HSPCs for the purpose of

stimulating CTLs via CEF peptide presentation. The use of CEF peptides in our CTL assays was supported by the results from our ELISpot experiments. Firstly, despite the wide range of variability in CTL response in healthy donors during CEF peptide incubation with PBMCs, all donors elicited a CTL response to CEF, confirming evidence in literature that > 90% of the adult population will have a memory CTL response to one of the CEF peptides within in the viral peptide pool (Sauerbrei et al, 2009; Evans & Niederman, 1989; Staras et al, 2006). This validation step was important to establish CEF peptides as a positive control for CTL stimulation in our assay where responses must be donor independent. Second, ELISpot data confirmed that CEF peptides had specificity for CTL stimulation and this was supported by the lack of response following CTL subset depletion from PBMCs. The next step was to prove that a CTL response could be elicited during CEF peptide incubation with platelets and further co-culturing with CTLs. Previous studies have shown that platelets can load peptides on MHC class I and present peptide antigens to $CD8^+$ T cells triggering activation (Chapman et al, 2012). Our studies did not show a significantly increased CTL response when CTLs were cultured with platelets and CEF peptides compared to CTL/CEF co-cultures. This was surprising as platelets are known to carry significant numbers of MHC class I molecules on their surface (Semple et al, 2011). A smaller CTL response, as measured by IFN-γ release was speculated to be due to platelets "mopping up" excess cytokines. In spite of these results, studies in literature encouraged us to incuabte megakarocytes and HSPCs with CEF peptides to elicit a CTL response, as platelets and megakaryocytes share many of the same receptors.

ELISpot data was also used to assess the effectiveness of CTL stimulation induced by anti-CD3 and CEF peptide incubation with CTLs in isolation from other PBMCs. In our study, $CD8^+$ T cells incubated with anti-CD3 or stimulated through CEF peptide presentaiton did not elicit as strong of a response as expected. This could be attributed to the the absence of sufficient co-stimulation from other PBMCs. Therefore, it was unknown how efficient CTL stimulation would be in the presence of the stimulating agent and megakaryocytes.

5.3 THE IMPACT OF FREEZE-THAW ON ANTI-CD3 AND PEPTIDE INDUCED $CD8^+$ T CELL STIMULATION

In the present study, PBMC, PBMCs depleted of $CD8^+$ T cells and $CD8^+$ T cell populations were tested in the ELISpot after freeze-thaw. The thawing protocol consisted of an overnight rest of all cell populations before being stimulated in the ELISpot. Our results support recent research that encourages an overnight rest period after thawing to improve CEF peptide induced $CD8^+$ T cell stimulation (Santos et al, 2015). While several studies do not perform an overnight rest of CTLs due to increased cell apoptosis and loss of cell numbers, Santos et al argue that resting T cells after thawing aids in promoting a tissue like state of cells with improved responsiveness to antigen (Santos et al, 2015). In our studies CTL viability after overnight rest was greater than 90% and $CD8^+$ T cell reactivity to CEF peptides and anti-CD3 was not compromised after freeze-thaw procedures. Therefore, procedures for cryopreservation and the overnight rest of CTLs after thawing was incorporated into assay development.

5.4 MEASURING HEALTHY DONOR MEGAKARYOCYTE APOPTOSIS IN THE PRESENCE OF STIMULATED CD8$^+$ T CELLS

The study of ITP pathophysiology has largely been focused on the impact of autoantibodies and cytotoxic T cells (CTLs) on platelet destruction. More recently, this focus has broadened to investigate the role of autoantibodies and CTLs in megakaryocyte development and platelet underproduction. Autoantibody mediated inhibition of megakaryocyte development and apoptosis has previously been examined (Yang et al, 2010; Chang et al, 2003). Contrarily, the impact of CTLs on megakaryocyte development and apoptosis in ITP has only been assessed in a single publication, where mixed mononuclear cell (MNC) cultures with or without CTLs were used to grow megakaryocytes (Li e al, 2007). For this study, we developed a new culture system where healthy donor PB derived CD34$^+$ HSPCs were cultured into mature megakaryocytes and further co-cultured with or without autologous CTLs in the presence of anti-CD3 or CEF peptides.

During TCR stimulation, CTLs are known to release cytokines such as IFN-γ and TNF-α (Anderson et al, 2006). While IFN-γ promotes the MHC class I presentation pathway and increases Fas proteins in the cell to encourage Fas mediated cell lysis, TNF-α signaling can trigger the caspase cascade in target cells, eventually resulting in cell apoptosis (Anderson et al, 2006). Therefore, we hypothesized that megakaryocyte apoptosis in healthy controls would be increased during co-culture with stimulated CTLs. The study design was optimized to observe megakaryocyte apoptosis in CTL/megakaryocyte co-cultures instead of comparing apoptosis in megakaryocyte co-

cultures of PBMCs and PBMCs depleted of the $CD8^+$ T cell subset. While the latter method was hypothesized to be more physiologically relevant, it is speculated that the PBMC population absorbed large amounts of the annexin-V stain resulting in low absorption by megakaryocytes in our studies. This, in part, may account for the absence of positive signals for increased annexin-V labeling on megakaryocytes during incubation with stimulated CTLs.

During *in-vitro* megakaryocyte/CTL co-culture, annexin-V labeling of $CD41^+$ cells cultured alone and during calcium ionophore incubation determined the range for the positive annexin-V signal to be between 44.0 ± 0.6% and 92.9 ± 5.6%. The level of annexin-V labeling on megakaryocytes cultured alone was higher than what was reported in healthy controls in the Li et al publication (26.46 ± 3.84% $CD41^+$annexin-V^+ labeled cells) (Li et al, 2007). One contributing factor of these observations may be that the HPSCs in our study were initially expanded with TPO and other cytokines and then subsequently stimulated with TPO a second time to promote megakaryopoiesis. Evidence shows that although TPO stimulation of $CD34^+$ HSPCs promotes megakaryocyte proliferation, it also promotes maturation *in-vitro* (Ryu et al, 2001). Therefore, it is speculated that the megakaryocytes in our study were very mature (81.2 ± 3.8% of cells were $CD41^+CD42^+$) and may have started to undergo thrombopoiesis. To reduce the number of apoptotic megakaryocytes in future studies and expand the range of a positive annexin-V signal, it may be beneficial to co-culture CTLs with megakaryocytes at an earlier time point in megakaryopoiesis.

Our preliminary results, in a single donor, showed no increase in megakaryocyte apoptosis during CTL co-culture with CEF peptide incubation. One possible explanation for the lack of increased megakaryocyte apoptosis in the "CEF" condition could be that this specific donor had a small frequency of CEF peptide specific CTLs, resulting in minimal TCR signaling and subsequently very little megakaryocyte apoptosis. The same donor showed increased megakaryocyte apoptosis in the presence of anti-CD3 stimulated CTLs. Unfortunately we cannot confirm megakaryocyte apoptosis in this study on the basis on annexin-V labeling alone. One of the main limitations of this study was the use of annexin-V as the only determinant of megakaryocyte apoptosis. Annexin-V is a marker of early cell apoptosis because it binds to phosphatidyl serine (PS), which gets expressed on the cell surface during apoptosis. The expression of PS as well as other markers of cell apoptosis including microvesiculation and loss of membrane integrity are also characteristic of platelet activation (Dale, 2005). Therefore, the use of annexin-V in the absence of additional markers for apoptosis prevented us from making conclusions about megakaryocyte apoptosis in this study. Platelet studies have shown that BH3 mimetics (anti-apoptotic protein inhibitors) can induce PS exposure on platelets that is Bak/Bax and caspase-dependent and is not impacted in the presence of platelet activation inhibitors (Schoenwaelder et al, 2009). More importantly, PS expression during platelet activation is not impacted by the inhibition of Bak/Bax and caspase activation. A study by White et al further supports these observations for megakaryocyte apoptosis (White et al, 2012). Inhibition of caspase 9, an initiator caspase, in megakaryocytes during BH3 mimetic treatment results in the inhibition of downstream caspase (3 and 7) activation and the

inhibition of apoptosis. To provide further confirmation, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) could also be used for labeling fragmented DNA in apoptotic megakaryocytes (Battinelli & Loscalzo, 2000). Therefore, in future studies it would be beneficial to investigate megakaryocyte apoptosis by not only looking at PS exposure, but to confirm that PS exposure is due to apoptosis and not activation by inhibiting Bax or caspase activation *in-vitro* as a control and to carry out TUNEL staining. Testing additional healthy donors would be required to determine whether CTL stimulation via anti-CD3 as well as CEF peptides can result in mature megakaryocyte apoptosis. It would also be beneficial to correlate levels of megakaryocyte apoptosis with markers of CTL cytotoxic activity such as granzyme B, which has been shown to have a significant role in CTL mediated platelet destruction (Zhou et al, 2014).

5.5 MEASURING INHIBITION OF MEGAKARYOCYTE GROWTH IN THE PRESENCE OF STIMULATED CD8$^+$ T CELLS IN HEALTHY DONORS

In this study we found that the presence of CTLs during *in-vitro* megakaryopoiesis did not impact the ability of CD34$^+$ HSPCs to commit to the megakaryocyte lineage (CD41$^+$) but was correlated to increased percentages of mature megakaryocytes (CD41$^+$CD42$^+$). This observation supports the use of a pure HSPC culture to initiate megakaryopoiesis instead of using a mixed mononuclear cell population previously used by Li et al, which may have had unknown impacts on megakaryocyte development. To develop a feasible CTL assay, it was necessary to confirm the use of flow cytometry technology to assess inhibition of megakaryocyte growth due to peptide specific CTL

stimulation using CD41 and CD42 markers. Similar to the megakaryocyte apoptosis assay we used two positive controls for $CD8^+$ T cell stimulation: anti-CD3 and CEF peptides.

All healthy donors showed inhibition of megakaryocyte growth during incubation with CTLs and anti-CD3. Surprisingly, inhibition in the presence of anti-CD3 stimulated CTLs was not always greater than with CEF peptide induced CTL stimulation. Instead of observing high levels of inhibition during anti-CD3 stimulation of CTLs, megakaryocyte growth was inhibited moderately, ranging from 30.1% and 53.9%. Consistent, moderate inhibition with anti-CD3 stimulated CTLs in all healthy donors supported the use of anti-CD3 as a positive control for our studies. During CEF peptide induced CTL stimulation, only 50% of healthy donors showed inhibition of megakaryocyte growth, which does not support the use of CEF peptides as a positive control for our studies. These results contradicted our initial hypothesis, which stated that despite variability to CEF peptide induced CTL stimulation almost 100% of the general population would show CTL reactivity. While this phenomenon was observed in all 5 healthy donors tested in our ELISpot experiments, it may be that other donors do not have CTL reactivity to CEF peptides. This would require the testing of additional donors. While it was not possible to confirm the absence or presence of donor CTL specificity to CEF peptides without carrying out the IFN-γ ELISpot, we can speculate that donors that did not show inhibition of megakaryocyte growth in the presence of CTLs and CEF peptides may have a very small frequency of CEF specific CTLs. This speculation was further supported by the fact that during CEF peptide induced CTL stimulation, donor A and B did not show increased megakaryocyte apoptosis nor inhibition of megakaryocyte growth. Another explanation

may be that CEF peptide induced CTL stimulation in literature is usually accomplished using PBMCs. Our studies stimulated CTLs by incubating them with TPO stimulated HPSCs co-cultured with CEF peptides, in the absence of other PBMCs. While it is common knowledge that naïve CTLs require antigen specific $CD4^+$ T helper cells to become activated and gain effector function, new research shows that effector memory CTLs also require T helper cells for full stimulation and cytokine release (Gao et al, 2002). So, while an individual donor may have showed CEF specific CTL activity in the ELISpot, donor CTLs incubated with HSPCs and CEF peptides may not have led to inhibition of megakaryocyte growth. This unfortunately was not confirmed as donors I and J, who showed relatively high CTL reactivity to CEF peptides in the ELISpot, did not yield minimal $CD34^+$ cell numbers after expansion to test in the megakaryocyte growth inhibition assay. Therefore, the ideal assay to test for CTL pathophysiology in platelet underproduction in ITP should use anti-CD3 as a positive control for CTL stimulation and not CEF peptides to ensure inhibition of megakaryocyte growth, independent of the donor.

The megakaryocyte growth inhibition assay was successfully retested in two out of four donors. The goal of re-testing was to assess whether CTL-mediated inhibition of megakaryocyte growth was consistent in donors overtime when CTLs were stimulated with CEF peptides or anti-CD3. In this study we were only able to observe the impact of CEF peptide incubation during individual HSPC/CTL co-cultures overtime. Both donors, G and A, were re-tested two months following initial testing. Donor G showed a moderate inhibition of megakaryopoiesis at both time points while donor A showed very minimal

inhibition. While CEF peptides may not be the ideal positive control for our assay, these results show that CEF peptides can be used as an internal control. These results are supported by findings in literature for stable CTL responses to the CEF peptide pool within individuals over approximately 3 years, as measured by the levels of IFN-γ and TNF-α in PBMCs using flow cytometry (Karlsson et al, 2007). For ITP testing, a single donor could be tested for CTL pathophysiology during active ITP, before and after treatment and during remission. As an internal control, the inhibition of megakaryopoiesis in HSPC/CTL co-cultures with CEF peptides should remain stable across all time points. Furthermore, these experiments proved that flow cytometry technology is sufficient to detect changes in megakaryocyte lineage commitment and maturity in the presence of peptide specific TCR stimulation of CTLs, opposed to 'peptide/MHC' mimicking anti-CD3 stimulation.

5.6 FUTURE DIRECTIONS

To further the development and validation of the assays in this study, it would be benefical to test additional healthy donors and initiate testing of ITP patients. Additionally, there are several other tests and assays that can be used to investigate the impact of CTLs on megakaryocyte development and/or apoptosis and platelet underproduction.

5.6.1 Measurement of Granzyme B in Megakaryocytes

Granzyme B was recently implicated in CTL mediated platelet destruction in ITP (Zhou et al, 2014). Furthermore this cytotoxic protein was reported to be increased in ITP

patients compared to healthy controls in CTL assays conducted in two separate studies (Olsson et al, 2003; Zhang et al, 2006). Recent studies in the field of CTL activity have discussed the limitations of measuring granzymes using anti-granzyme antibodies via flow cytometry including the uncertainty as to whether the measured granzymes are proteolytically active. One group has developed a new flow cytometry protocol involving a selective fluorogenic granzyme B substrate to measure granzyme B in the target cell (Packard et al, 2007). Additionally, the use of confocal imaging by Packard et al has shown that by using this method, granzyme B can be detected both in the target cell as well as at the edges between the target and effector cell supporting the presence of a synapse like region between the cells for CTL related protease activity. Therefore, it may be beneficial to use a flow cytometry and/or confocal microscopy protocol to measure cytotoxic protein markers indicative of CTL effector function in our target cells – megakaryocytes. This type of assay would increase the validity of results reported in the megakaryocyte apoptosis and growth inhibition assays and confirm CTL cytotoxicity specific for megakaryocyte peptide antigens.

5.6.2 CTL Mediated Platelet Apoptosis Assay

The underlying mechanisms that contribute to the heterogeneous pathology of ITP are still unclear and therefore it would be naïve to limit our studies to only cultured megakaryocytes and not platelets. Obtaining large numbers of platelets requires minimal technical skill and time compared to culturing megakaryocytes from peripheral blood. Designing a screen for ITP patients consisting of donor CTLs and platelets in a co-culture setup and assaying for platelet apoptosis would be ideal. Platelet studies should be carried

out with multiple markers to investigate apoptosis such as a combination of annexin-V and the presence of caspase activation, to ensure that in-vitro studies can confirm platelet apoptosis and not just activation. Platelets share many of the same protein receptors found on megakaryocytes and therefore, platelet apoptosis in the presence of CTLs may be a good indicator of those patients that would show high levels of apoptosis when ITP megakaryocytes are incubated with autologous CTLs. By extending our studies to include platelet/CTL co-cultures, we may also be able to uncover whether platelet destruction and underproduction can be mediated by CTLs in the same donor.

6.0 CONCLUSION

The exact immune mechanisms that underly cytotoxic T lymphocyte (CTL) mediated platelet destruction and/or underproduction in ITP have yet to be confirmed. Our results have shown that hematopoeitic stem and progenitor cells can be isolated from small volumes of peripheral blood, expanded *ex-vivo* and further cultured to form mature megakaryocytes. A megakaryocyte growth inhibition assay was developed and showed that the presence of CTLs during culture of TPO stimulated HSPCs does not impact megakarycoyte lineage commitment or maturity. Future studies should stimulate CTLs with anti-CD3 during *in-vitro* megakaryopoeisis as a positive control to induce inhibition of megakaryocyte development, with the option of including CEF stimulated CTLs as an internal control. Further research needs to be carried out to determine whether anti-CD3 incubation with CTLs can impact megakaryocyte apoptosis in additonal healthy donors and to determine the appropriate testing controls and additional markers to confirm megakaryocyte apoptosis *in-vitro*. The assays outlined in this thesis have a potential role in being extended to ITP patient samples in the McMaster ITP longitudinal study and may be used to study patients over time. It will however, be necessary to determine whether HSPCs from ITP patients can be grown into mature megakryocytes, as seen with healthy controls. Patients who show inhibition in megakaryocyte development or increased megakaryocyte apoptosis during CTL incubation with TPO stimulated HSPCs would indicate CTL specificity for megakaryocyte autoantigens.

Investigation supporting CTL mediated platelet underproduction in ITP can have tremendous value in clinical practice. While the assays in this study are in the early stages

of development, they may prove useful to assess the efficacy of response to certain ITP therapies aimed at decreasing autoimmune activity in ITP due to T cells. There is potential to develop a feasible screen for autoreactive CTL activity using platelets if patients that exhibit CTL mediated megakaryocyte apoptosis or growth inhibition also exhibit CTL mediated platelet destruction. While changing the clinical management of a patient that tests "positive" for autoreactive CTL activity is naïve, we may start to investigate the clinical profiles and antibody-testing data of these patients compared to patients that test "negative".

Furthermore, the assay may also be extended to study the impact of ITP autoantibodies (serum) on autologous HSPCs and could provide insight into whether patients can be pigeonholed as having CTL mediated ITP versus antibody mediated ITP. This can be beneficial in two ways, one, antibody impacts on megakaryopoiesis in ITP are currently carried out in a non-autologous fashion and two, if we can begin to characterize individual ITP cases and compare findings at the "bench" to clinical and antibody-testing data, it may help us better characterize the immune mechanisms that underlie this disease. Moreover, this assay is not only limited in its usefulness to study immune mechanisms in ITP but could also be extended to study other thrombocytopenic disorders due to its autologous nature and feasible laboratory practices.

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