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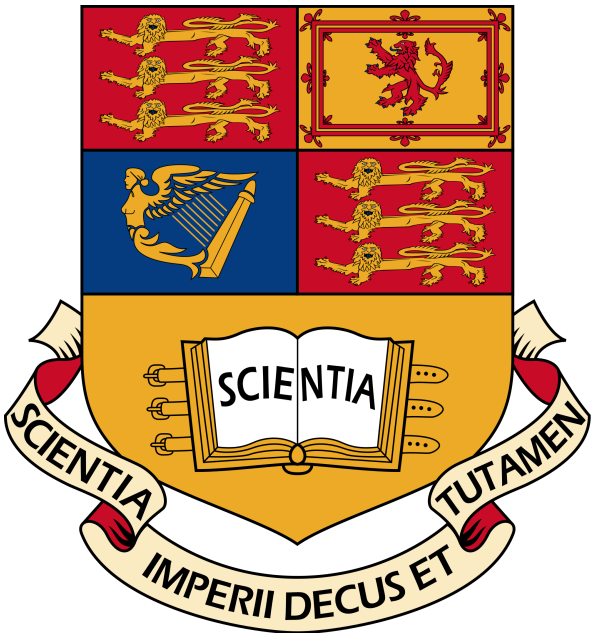
Exploring novel inhibitory mechanism on megakaryopoiesis in immune thrombocytopenia



Luis Edgardo Gonzales Huerta
CID 01129296

"A thesis submitted in partial fulfilment of the requirements for the degree of MSc in Immunology and for the Diploma of Imperial College London"

Imperial College London
September 2016

Dr. Nichola Cooper
Dr. Anwar Sayed

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Nichola Cooper. Her support, guidance, trust and example are lessons that I will carry beyond the MSc course.

I am also deeply grateful to Dr. Anwar Sayed and Dr Ahmad Khoder, whom I had the privilege to work with. The invaluable knowledge they shared with me made the completion of this thesis possible.

Very special thanks to my dear friends Denisse García, Leon de Boer, Ralf Wenz, Youhani Samarakoon and Giuliana Mastropietro, from whom I learned and owe finding a home away from my country.

Also, I would like to express gratitude to Alice Glaser, Tom Mayo, Daniel Ang and the staff and students of the Haematology Centre, Imperial College London.

Thank you to Dr. Sophie Rutschmann and Dr. Neil Galloway for providing a space to carry out with my work when circumstances got difficult.

Finally, to my father, Raúl, who made this possible for me and to whom I owe every achievement in my life.

Table of content

Abbreviations

A	
APCs	Antigen presenting cells
APC	Allophycocyanin
APRIL	a proliferation-inducing ligand
B	
BAFF	B-cell activating factor
BCR	B cell receptor
C	
CD	Cluster of differentiation
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CTL	Cytotoxic T cells
CTLA - 4	CTL-associated antigen 4
D	
DAPI	4,6-diamidino-2-phenylindole
DC	Dendritic cells
DMSO	Dimethyl Sulfoxide
E	
ECT2	Epithelial cell-transforming sequence 2
F	
Fabs	Fragment antigen-binding
FBS	Foetal Bovine Serum
FcR	Fraction crystallisable receptor
FITC	Fluorescein isothiocyanate
FLI-1	Friend-leukemia integration 1
Flt3	FMS-like tyrosine kinase 3
FMO	Fluorescence minus one
G	
GDP	Guanine diphosphate
GEF-H1	Guanine nucleaotide exchange factor
GMP	Granulocytes/macrophages progenitors
gp	Glycoproteins
GTP	Guanine triphosphate
H	
HLA	Human Leukocyte antigen
HSC	Haematopoietic Stem Cells
I	
IDO1	Immunomodulatory enzyme indoleamine2,3-dioxygenase1
IFN-γ	Interferon gamma
IgG	Immunoglobulin gamma
IgM	Immunoglobulin M

IL	Interleukine
ITP	Immune Thrombocytopenia
IVIg	Intravenous immunoglobulins
K	
KLF-1	Krueppe-like factor 1
L	
LSK	Lin Sca-1 c-kit
LT-HSC	Long term HSC
M	
MEP	Megakaryocyte/erythrocytes progenitors
MHC I	Major histocompatibility complex class I
MHC II	Major histocompatibility complex class II
MK	Megakaryocytes
MKP	Megakaryocyte progenitors
MPP	Multipotent progenitor
MYH10	Myosin IIB heavy chain
N	
NF-κβ	Necrosis factor kappa beta
NK	Natural killer cells
P	
PD-1	Programmed death-1 receptor
PD-L1	Programmed death ligand 1
PE	Phycoerythrin
PECy7	Phycoerythrin-Cyanine 7
PerCP	Peridinin chlorophyll
PI3K	Phosphoinositide 3-Kinase
R	
RUNX-1	Runt related transcription factor 1
S	
SD	Standard deviation
ST-HSC	Short term HSC
T	
TCR	T cell receptor
TNF-α	Tumour Necrosis factor alpha
TNF-β	Tumour Necrosis factor beta
TPO	Thrombopoietin
TRA	Thrombopoietin receptor agonists
V	
vWF	von Willebrand factor

Abstract

Megakaryocytes (MKs) derive from haematopoietic stem cells (HSCs) by a process called megakaryopoiesis. One of the main functions of MK is the production of platelets. Immune thrombocytopenia (ITP) is defined as a reduction in the platelet count below 100 x 10^9/L in peripheral blood in the absence of a clear cause. It has been suggested that T cells are primed against MK. Our aim is to determine whether plasma of ITP patients alters characteristics of megakaryopoiesis including terminal development and expression of immune markers including PD-L1 (CD274) and HLA-DR.

CD34+ HSC were cultured for 10 days to differentiate them into MKs. At day 7, plasma from 3 healthy donor and 4 ITP patients were added. Study of expression of CD34, CD38, CD41, CD42b, CD274 and HLA-DR was performed with flow cytometry. Confocal microscopy was performed to confirm the expression of PD-L1 throughout the development.

Culturing the cells with healthy control plasma resulted in significantly higher number of $CD42^+CD41^+CD38^+$ cells, as well as significant lower expression of HLA-DR compared to medium only and ITP patients' plasma. . In contrast, MKs cultured in plasma from patients with ITP had a higher HLA-DR expression than MKs cultured in healthy plasma, demonstrating an important role for plasma derived factors to alter immune regulatory potential for MKs and platelets. PD-L1 expression was positive throughout the development but it did not change significantly with the co-culture with either healthy control or ITP patients' plasma, compared to medium only.

MKP and mature MK can express PD-L1 *in vitro* culture. Also, healthy plasma and ITP plasma has the potential to enhance megakaryopoiesis.

Key words: Megakaryocytes, ITP, CD274, PD-L1, HLA-DR.

Word count: 8540

Chapter I. Introduction

I.1 Megakaryocytes

It is important to clarify that our understanding of the developmental process has multiple gaps and, a substantial amount of our knowledge derives from research in murine models, which does not apply necessarily to humans (Gabdoulline et al., 2015; Pera & Trounson, 2004; Woolthuis & Park, 2016). Nevertheless, the most spread model conceives the hierarchical concept based on HSCs' loss of self-renewal and pluripotency (Woolthuis & Park, 2016).

The classical model description starts with the loss of self-renewal capacity in HSCs to give rise to multipotent progenitors (MPPs), which maintain pluripotency. Further differentiation will provide cells with a restricted lineage potential, known as common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). CMPs are accountable for megakaryocytes/erythrocytes progenitors (MEP), granulocytes/macrophages progenitors (GMP) and dendritic cell (DC) progenitors. On the other hand, CLP are responsible for T cell progenitors, B cell progenitors, NK cell progenitors and DC progenitors (Mosaad, 2014; Woolthuis & Park, 2016) (Figure 1).

The model for megakaryopoiesis is a work in progress. In recent years, some murine studies have shown the presence of new progenitor populations and, an unexpected wide differentiation capacity in them. This progress in the model of MKs differentiation has been based on the characterisation of multiple markers: Lin Sca-1 c-kit (LSK), CD34, CD38, CD41, CD42b, CD61, CD110, von Willebrand factor (vWF) and FMS-like tyrosine kinase 3 (Flt3)(Adolfsson et al., 2001, 2005; Woolthuis & Park, 2016; Yang et al., 2005). A brief description of those markers is given in Table 1 (Furness & McNagny, 2006; Levis & Small, 2003; J. Li, Xia, & Kuter, 2000; Shrimp et al., 2014; Zwicker, Lacroix, Dignat-george, Furie, & Furie, 2012).

In the 1990s, several studies were done in mice to identify markers for the most primitive progenitor in haematopoiesis. By the end of the decade, the recognition of Lin^{-} $Sca\text{-}1^{+}c\text{-}kit^{+}CD34^{-}$ ($LSKCD34^{-}$) as long-term HSCs (LT-HSCs) and $Lin^{-}Sca\text{-}1^{+}c\text{-}$

kit^{+}CD34^{+} (LSKCD34^{+}) as short-term HSCs (ST-HSCs) was established for the murine model (Nakauchi, Takano, Ema, & Osawa, 1999; Sato, Laver, & Ogawa, 1999).

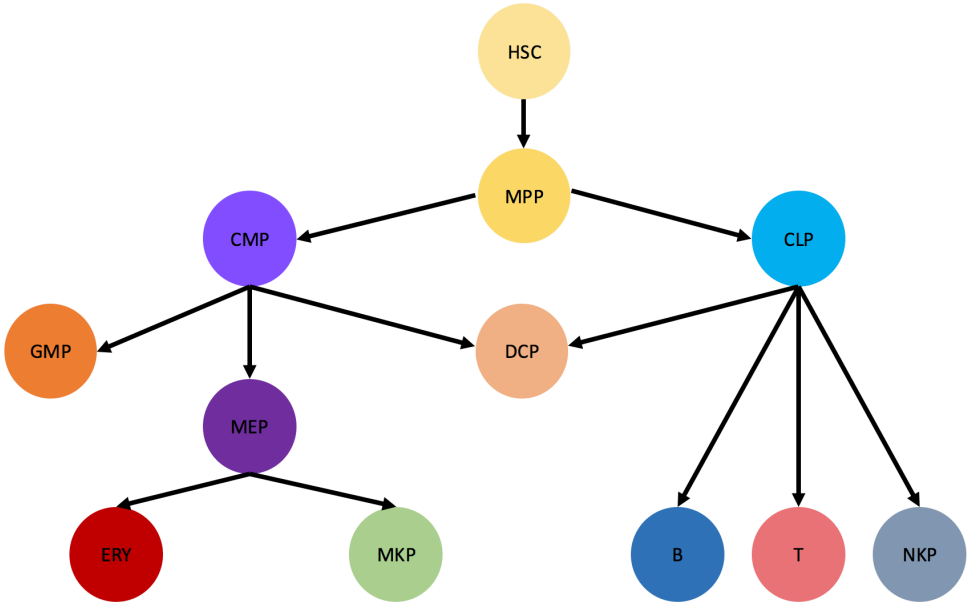


Figure 1. Classical model of differentiation of HSC. It describes the versatility of a pluripotent HSC to produce most lineages' progenitors in a 5 stages hierarchical structure.

The characterisation of human HSC's surface markers took longer. Since the 1960s, CD34 was considered as a reliable marker for HSCs in the bone marrow. Cells with surface expression of CD34 show high capacity for differentiation into multiple lineages (Krause, Fackler, Civin, & May, 1996). Furthermore, its presence conjointly with other markers, allow to establish an early progenitor, because as development progresses, CD34 is downregulated (Mosaad, 2014). However, Goodell et al, developed an experiment based in Hoechst dye efflux activity, and identified that some CD34^{-} cells were HSCs, establishing them as long-term culture initiating cells. Also, they demonstrated that these cells were the responsible to give rise to CD34^{+} (Goodell et al., 1997; Guo, Lubbert, & Engelhardt, 2003).

Later, Adolfsson et al. revealed that the loss of self-renewal in LT-HSCs matched the upregulation of Flt3 (Adolfsson et al., 2001). This finding turned out to be relevant in the description of cells throughout their differentiation, since, to a certain extent, the regulation of Flt3 is predictive of lineage predilection (Woolthuis & Park, 2016).

A descriptive study in mice, based on the regulation and $CD34^-$ fraction of Lin^- $Sca\text{-}1^+c\text{-}Kit^+$ cells presented a preference in $LSKCD34^-Flt3^-$ and $LSKCD34^+Flt3^-$ towards MK development. On the other hand, $LSKCD34^+Flt3^{hi}$ cells exhibited a limited capacity to differentiate into MEP (Adolfsson et al., 2005; Woolthuis & Park, 2016) (Figure 2).

MKs and erythrocytes rise from a common progenitor, hence it is important to remark that further differentiation into MK will require the interaction of two transcription factors: Friend-leukaemia integration 1 (FLI-1) and Runt related transcription factor 1 (RUNX-1) (Eto & Kunishima 2016; Okada et al. 2013). Even though, FLI-1 is a crucial actor, it seems that it has an inevitable dependency of RUNX-1 to inhibit the dephosphorylation of serine 10 (Draper et al., 2016; Huang et al., 2009; Okada et al., 2013). Additionally, RUNX-1 blocks Krueppel-like factor 1 (KLF-1), the erythroid master regulator, leaning the balance towards FLI-1 and, therefore, MK differentiation (Kuvardina et al., 2015). Additionally, the gene DLX4 has shown predilection for MK differentiation. According to Trinh et al., using gene set enrichment analysis, DLX4 seems to induce shift towards independent MK gene sets. They showed evidence of DLX4 upregulating IL-1β through activation of NF-κβ (Trinh et al., 2015) (Figure 3).

Table 1. Surface markers in HSCs and MKs.

Marker	Alternate name	Description
Lin	Lineage marker	Consists in every molecule and cluster that might allow the identification of a determined pathway of differentiation.
Sca-1	Ly6A/E Stem Cell Antigen - 1	Cysteine-rich glycosyl phosphatidylinositol-anchored cell surface protein, localized in lipid rafts of cells' membranes. Role in cell signaling.
c-kit	CD117	Tyrosine kinases receptor. Transmembrane protein. Receptor for Stem Cell Factor (SCF). Important role in multiple cellular activities as differentiation.
CD34	Mucosialin	Transmembrane glycoprotein in early HSC. No established function, it has been associated to inhibition of differentiation, proliferation in HSCs, intracellular signaling and adhesion properties.
CD38	Cyclic ADP-ribose (cADPR)	Transmembrane glycoprotein. Functions are associated to metabolism of Nicotinamide adenine dinucleotide (NAD).
CD41	Glycoprotein α IIb (gpIIb) Integrin α2b	Transmembrane glycoprotein. Produces a complex with CD61 to perform adhesion functions.
CD42a	Platelet Glycoprotein IX chain (gpIX)	Transmembrane glycoprotein associated to platelet activation and adhesion.
CD42b	Platelet Glycoprotein Ib α chain (gpIb)	Transmembrane glycoprotein that expresses lectin-binding oligosaccharides.
CD61	Glycoprotein IIIa (gpIIIa) Integrin β3	Transmembrane glycoprotein. Produces a complex with CD41 to perform adhesion functions.
CD110	c - Mpl TPO high affinity receptor	Receptor present in HSCs that induces MK differentiation and platelets production.
von Willebrand factor (vWF)	Factor XIIIvWF	Glycoprotein in bloodstream that acts as a adhesion structure for platelets.
FMS-like tyrosine kinase 3 (flt3)	CD135	Receptor of tyrosine kinase. Related to lymphocytic lineage.

Figure 4 shows differentiation pathway and expression of surface markers. During the final stage of platelets production, endomitosis starts, which implies the accumulation of DNA content in a single polylobulated nucleus without cytokinesis (polyploidization). This process requires the down-regulation of myosin IIB heavy chain (MYH10) in the contractile ring by RUNX1 (Eto & Kunishima, 2016; Okada et al., 2013). Further membrane changes get altered and cytokinesis blocked by down-regulation of the microtubule-associated guanine nucleotide exchange factor (GEF-H1) and epithelial cell–transforming sequence 2 (ECT2), which modulated RhoA activity in the cell membrane. RhoA is an enzyme that induces the synthesis of guanine triphosphate (GTP) from guanine diphosphate (GDP) and is determinant in cytokinesis, since it induces cytoskeleton rearrangement and myosin activity (Gao et al., 2012) (Figure 5).

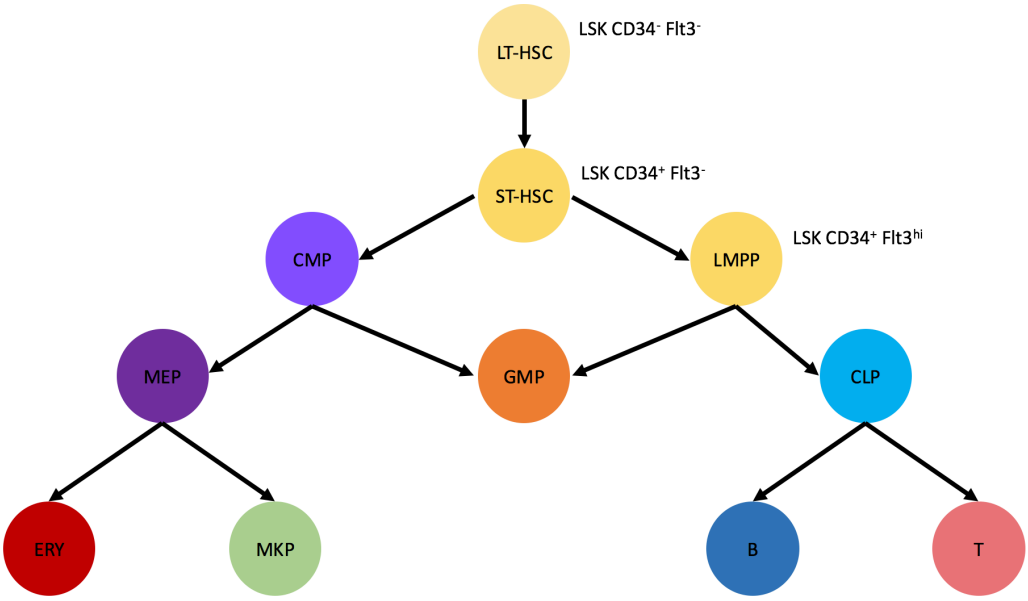


Figure 2. Current murine model of HSC differentiation. From top to bottom, HSCs are divided into LT-HSC and ST-HSC subsets, according to their capacity for self-renewal. Identification of both subsets is determined by their expression of LSK, CD34 and Flt3. LT-HSCs are shown as the most primitive progenitor, with the potential to give rise to all different lineages in 5 stages of differentiation.

The final purpose of MKs is the production of platelets through a process called thrombopoiesis. Endomitosis outcome are MKs with a high ploidy, that will develop pseudopods, cytoplasmatic extensions known as proplatelets. Granules containing RNA and organelles as mitochondria move to the end of the pseudopods that extend to vessels, where circulation finally detach platelets into the bloodstream (Deutsch & Tomer, 2006).

Thrombopoietin (TPO), which is produced in the liver by hepatocytes as a consequence of IL-6 stimulation and in the stromal cells of the bone, help in development

of MKs. In normal conditions, it seems that TPO levels keep a steady production. The concentration in plasma would be affected by the amount of platelets and MKs, which consumes TPO through c-Mpl receptors. Therefore, a high amount of platelets implies a low level of TPO and less MKs differentiation. On the other hand, a low amount of platelets would imply less consumption of TPO and more induction of MKs differentiation (Grozovsky, Giannini, Falet, & Hoffmeister, 2015). Nevertheless, this model has not been fully characterized and accepted since it fails to predict the evolution of certain pathologies, such as ITP. Kosugi and col., demonstrated that concentration of TPO in ITP patients was only mildly elevated in 70% of the cases. In 30% of the patients the levels of TPO were equal to healthy controls (Kosugi et al., 1996).

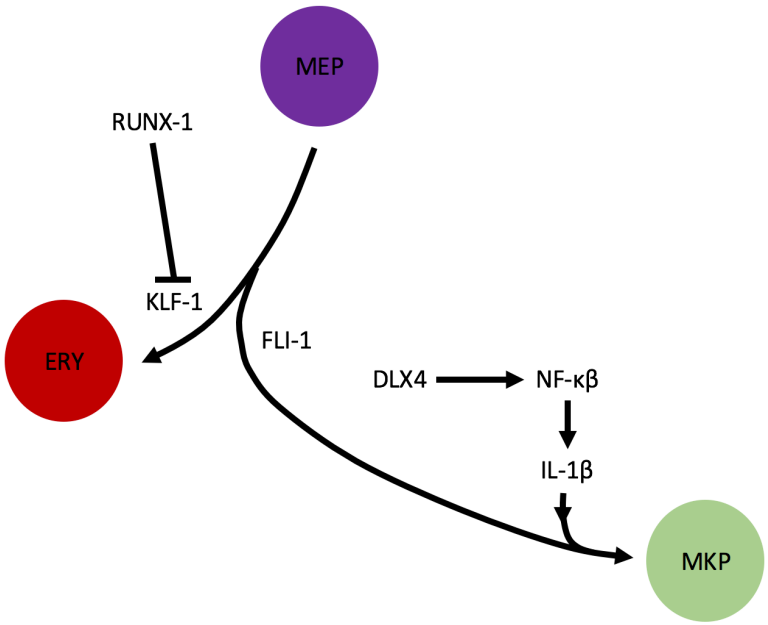


Figure 3. Transcription factors involved in final stages of MKP differentiation. MEP predilection to differentiate into erythroid progenitor or MKP will translate to a well described dynamic of transcriptional factors. MKPs will require inhibition of KLF-1 by RUNX-1, upregulation of FLI-1 and activation of DLX4 gene, with subsequent NF-κβ expression and IL-1β stimulation.

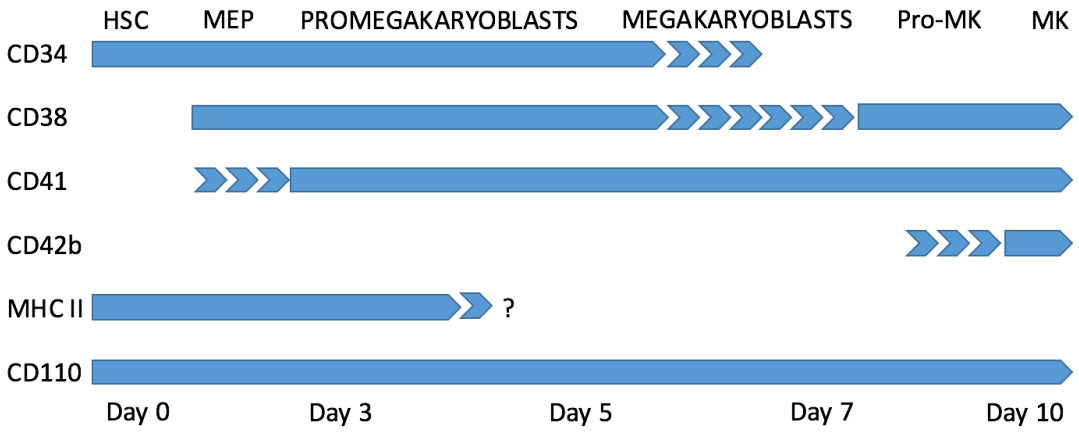


Figure 4. Megakaryocyte developmental pathway (Attar, 2014; Tomer & Harker, 1996)**.** Six surface markers, that help identify specific developmental stages, have been represented in the figure. Continuous line represent expression on whole population. Discontinuous line implies uneven presence of the specific marker. Time points (Day 0, 3, 5, 7 and 10) have been proposed under laboratory conditions, nevertheless, it should not be considered as representation of normal physiological development. The loss of expression of MHC II in MKP has not been described in detail before, thus the exact moment it disappears cannot be represented. A mature MK should express CD38, CD41, CD42b and CD110 in its surface.

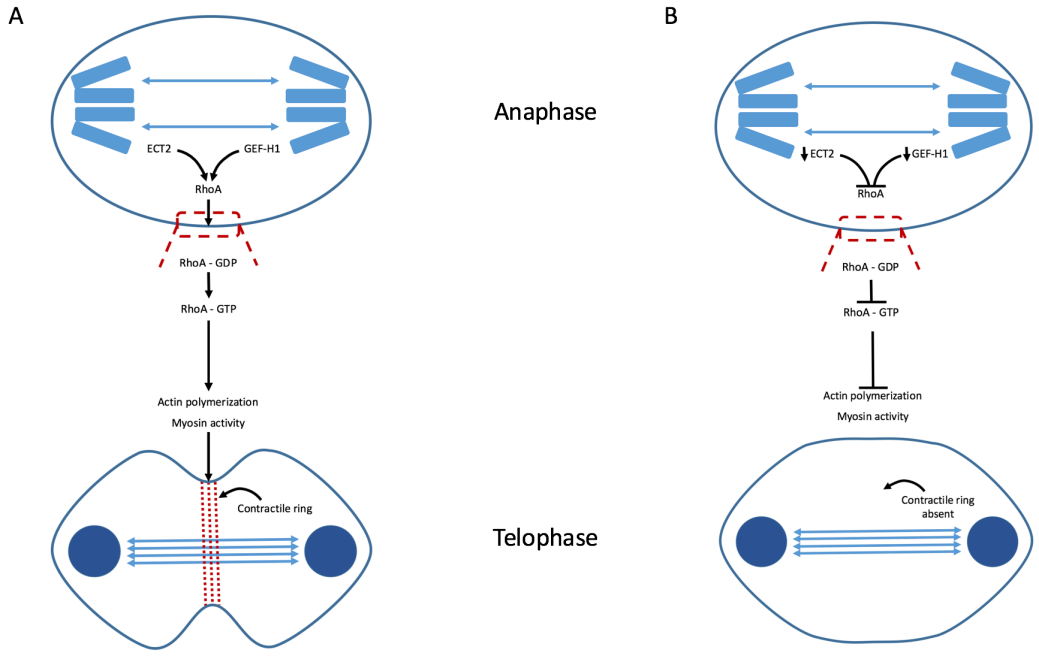


Figure 5. MKs endomitosis. Figure A, shows the action of ECT2 and GEF-H1 on RhoA in anaphase cell cycle, for development of the contractile ring and posterior formation of a cleavage furrow in telophase. Figure B, represents the differences of this process in MK maturation. Gao et al, demonstrated a downregulation of ECT2 and GEF-H1, leading to restriction of cell division and promoting polyploidization in MKs.

I.2. Immune Thrombocytopenia (ITP)

I.2.1 Overview

ITP is a condition characterized by the significant reduction of platelets. Although considered to be an autoimmune disease, there is no clear evidence of a specific cause in all patients. In the overall population, the incidence of ITP is 4/100000 and the prevalence is around 24/100000. (Provan & Newland, 2015; Schoonen et al., 2009) with a higher incidence in women. Otherwise there is no clear characterisation of a vulnerable population for ITP. Additionally, the lack of evidence for the cause of ITP makes it difficult to build a model of the disorder, and can even lead us to think that we are facing more than one disease with thrombocytopenia as the end result of a number of different pathologies. The significant reduction of platelets seen in patients with ITP is related to an increased risk of bleeding (Provan & Newland, 2015; Provan et al., 2010; Rodeghiero et al., 2009) although the severity of the bleeding is rarely lethal (Portielje, 2001).

I.2.2 Diagnosis

The diagnosis of ITP is mainly based on the clinical history and the decrease of platelet count below 100 x 10^9/L in peripheral blood in the absence of an evident cause. Presence of self-antibodies are not considered in the diagnostic criteria since 30 -40% of ITP patients do not show positivity in antibodies assays. Also, it has been shown that autoantibodies can be identified in cases of non-immune thrombocytopenia, giving a specificity of 87.5% (R McMillan, Wang, & Tani, 2003; Provan et al., 2010).

I.3 Immunological abnormalities in ITP

I.3.1 Overview

Current understanding of ITP pathogenesis is commonly characterized by peripheral tolerance defects, differentiation blocks with skewed peripheral B-cell subsets and central tolerance defects (Kistangari & McCrae, 2013). The break of tolerance leads in some patients to production of autoantibodies (IgG) that target GPIIIa/IIb and GPIb/IX. These glycoproteins are present in the membrane of MKs and of platelets that are

endocytosed by macrophages in the spleen and liver, via fraction crystallisable gamma receptors (FcγR). (Kistangari & McCrae, 2013; McCrae, 2011; Toltl, Nazi, Jafari, & Arnold, 2011). However, some abnormalities in T cells and MKs development have been established (X. Ji, Zhang, Peng, & Hou, 2014). Also, a new mechanism has been recently described, involving a dysregulation of sialic acid in platelets surface, has been suggested as a contributing factor (Shao et al., 2014).

I.3.2 B-cells

B cells obtain tolerance for self-antigens in the bone marrow. Immature B cells, expressing immunoglobulin M (IgM) B cell receptor (BCR) get exposed to self-antigens and, in case of reactivity, most of them will get eliminated from the pool of immune cells. Under normal circumstances 55 - 75% of immature B cells will show specificity for self-antigens. This percentage will drop to 20 - 40% in transitional and mature B cells (Pelanda & Torres, 2012). The regulatory mechanisms involved in the inhibition of these cells are broad and include cell deletion and receptor editing. (Toltl et al., 2011).

Cell deletion is triggered when a B cell shows affinity to self-antigen. This process requires the active role of Bcl-2 molecule and the downregulation of two growth factor: B-cell activating factor (BAFF) and of a proliferation-inducing ligand (APRIL). In ITP, evidence of upregulation of both B cells growth factors has been shown (Emmerich et al., 2007; Gu et al., 2009).

Continuous receptor editing in B-cells, operates as a safety mechanism to prevent persistence of self-reactive immune cells. In that respect, some studies were performed to elucidate VDJ recombination characteristics in ITP patients. Although, it has been established that there is an overexpression of VH6 gene, it has not been associated directly with ITP pathogenesis (van Dijk-Hard, Feld, Holmberg, & Lundkvist, 1999).

The production of specific autoantibodies implies the activation of particular mechanisms of the immune system as germinal centre formation, somatic recombination and class switch of B-cells. Moreover, it has been suggested that it is a driven by specific antigen stimulus. Roark et al. presented several antibodies' features to support this claim, which included a single heavy chain V_HDJ_H rearrangement, high replacement to silent

ratio, and platelets reactive fragment antigen-binding (Fabs) that originated from IgG library. These evidences imply derivation from a single B-cell and isotype switching (Roark, Bussel, Cines, & Siegel, 2002).

Despite, the evidence of B-cells involvement in ITP pathogenesis, there are a lot of gaps to clarify. 30 to 40 % of patients do not have anti-platelets' antibodies (Robert McMillan, 2003). Also, whether B-cells disorder is a starting point or a consequence of something else, is still a matter of debate.

I.3.3 T-cells

The tendency for $CD4^+$ Th_0 - Th_1 cells cytokines in ITP patients, is evidence of cellular immunity involvement. Patients with chronic ITP show a significant increase of IL-2, interferon gamma (IFN-γ) and Tumour Necrosis Factor beta (TNF-β) (X. Ji et al., 2014; Kuwana & Ikeda, 2005; J W Semple, 2013; Toltl et al., 2011). Furthermore, clear evidence has been established of an increased Th_1/Th_2 ratio (Cooper & Bussel, 2006; Robert McMillan, 2007).

$CD4^+$ CTLs is a small subset of T cells with capacity to produce cytotoxicity through the release of granzymes and perforines, characteristics mainly associated to $CD8^+$ T cells. $CD4^+$ CTLs represent around 2% of all T cells in humans, and their role is usually associated to immune response against viral infections that affect antigen presenting cells (APCs) (Appay, 2004; Marshall & Swain, 2011). Nevertheless, some evidence suggests more plasticity from these cells activity, showing capacity to act against cancer cells. Furthermore, Quezada et al. showed that the blockage of the CTLA-4 molecule boosts $CD4^+$ CTLs expansion (Quezada et al., 2010). This detail becomes relevant for ITP studies since it has been showed that it produces a decreased level of CTLA-4 (Zhu et al., 2015).

Ye et al. revealed a significant role of a pathway composed by IL-23 and Th_{17} cells studying 30 cases of acute ITP. IL-23 has a pro-inflammatory activity and is closely related to IL-12. An increased expression of IL-23, IL-17 and of receptors for IL-23 (IL-23R / IL-12Rβ1) were detected. Th_{17} is a subset of T-cells that has pro-inflammatory functions and their increased presence in ITP patients correlated to IL-23. Finally, the

authors suggested that a boost of Th_{17} activity might be important in the pathogenesis of ITP. (Ye et al. 2015). These findings were supporting the previous data reported by Ji et al. (L. Ji et al., 2012) that showed a decrease in T_{reg}/Th_{17} ratio in ITP patients. T_{reg} are cells that induce immune tolerance and are strongly related to the prevention of autoimmune diseases. The decrease of T_{reg} was further studied Catani et al. finding downregulation of the immunomodulatory enzyme indoleamine 2,3-dioxygenase 1 (IDO1) in DCs (Catani et al., 2013).

T-cell activation is a complex process that requires multiple conditions to be fulfilled to happen. It is mainly triggered by the break of balance of multiple activation and inhibitory signals. For example, $CD8^+$ T cells' killing activity will require presentation of antigen by the major histocompatibility complex (MHC) class I to TCR and the binding of CD8 with MHC class I $\alpha 3$ domain (Belyakov et al., 2007), which is considered as signal 1 in a three signals requirement. Signal 2 is established by the stimulation of CD28 by CD80 and CD86 (Ertelt et al., 2013). Finally, the presence of specific cytokines such as INF-γ will be signal 3. Additionally, to the activation triad, inhibitory receptors as CTL-associated antigen 4 (CTLA-4) molecule (Tivol et al., 1995) or Programmed Death-1 receptor (PD-1) in the surface of T-cells need to decrease their intracellular signaling (M E Keir, Butte, Freeman, & Sharpe, 2008). In the case of $CD8^+$ T cells, dynamics and activation has not been studied in detail in ITP patients.

Olsson et al. showed that there is an increase of $CD8^+$ T cells in peripheral blood and that that these cells would be primed against platelets and MKs in ITP patients. Furthermore, they presented data about immunosuppressive treatment inhibiting their killing activity (Olsson et al., 2003).

Whether we consider $CD4^+$ or $CD8^+$ T cells as important in ITP, MHC molecules will require to present an antigen to produce activation (Schmidt, Dojcinovic, Guillaume, & Luescher, 2013). Studies about this subject in ITP patients are limited. Although, some have suggested no relationship between specific MHC alleles and ITP (Gaiger, Neumeister, Heinzl, Pabinger, & Panzer, 1994), others consider plausible a disturbance in MHC presentation due to cytokines' effect (Chanock, 2003).

I.3.4 Inhibition of megakaryopoiesis in ITP

Although, antibodies against gpIIb/IIIa and gpIb/IX role has been considered as platelet targeting exclusively, some data has been shown suggesting an effect on normal MK's function. Chang et al, tested healthy donor's plasma and ITP patients' plasma, with evidence of anti-platelets antibodies, on MKs. The authors concluded that antibodies presence produces an inhibition of megakaryopoiesis (Chang et al., 2003). Other studies would support these conclusions (Robert McMillan, Wang, Tomer, Nichol, & Pistillo, 2004).

Although, several pieces of data reveal a complex disease that dysregulate most components of the immune system, there is no definitive aetiology or model of pathogenesis established. Figure 6 shows a scheme of the current knowledge about ITP.

I.4 Current treatments

Most treatment strategies have been targeted to inhibit immune mediated destruction of platelets. Immunosuppression is achieved through the use of corticoid steroids. Intravenous immunoglobulins (IVIg) and anti-D work by saturating Fc receptors, blocking endocitosis by macrophages and allowing platelets covered by antibodies to stay in circulation. Treatment with IVIg has been associated with decrease of IL-12, maturation of DCs and increase of IL-10 (Provan & Newland, 2015). Generally, these schemes provide remission in 80% of patients with ITP (Cooper, 2009).

Second line treatments include Rituximab and thrombopoietin receptor agonists (TRAs) (Neunert, Lim, & Crowther, 2011). Rituximab, a chimeric monoclonal antibody against B cell CD20, aims to decrease the number of plasma cells and, therefore, to deplete the manufacturing kit of autoantibodies. Treatment with Rituximab has shown increased T_{reg} activity in responders and normalization of other T-cell subsets' activity (Stasi et al., 2008). Nevertheless, only 50-60% of patients respond to treatment and long lasting effects seems to be restricted to a third of the cases and due to safety issues there is still much discussion about the suitability of this treatment (Arnold et al., 2007). On the other hand, TRAs have appeared in the scene as a treatment alternative. Romiplostim

and eltrombopag induce megakaryopoiesis and increase the production of platelets to compensate the peripheral depletion. TRAs mimic the activity of TPO, which by stimulation of the receptor c-MPL in the surface of HSC triggers the differentiation to MKs (Imbach & Crowther, 2011).

I.5 Programmed death ligand 1/Programmed death receptor 1 (PD–L1/PD-1)

PD-L1 and PD-1 have been established as regulatory molecules for central and peripheral tolerance (Al-Chaqmaqchi et al., 2013; D'Addio et al., 2011; M E Keir et al., 2008; L. Wang et al., 2008). Their expression in the thymus proved to be important in shaping T-cells maturation and selection (Mary E Keir, Latchman, Freeman, & Sharpe, 2005). Furthermore, they are protagonists in T-cell function (S. Wang et al., 2003).

The binding of PD-1 by PD-L1 induces the blockage of phosphoinositide 3-kinase (PI_3K), and subsequently, to the decrease cytokines production. The cytokines mainly involved in this pathway are IL-2, INF-γ and cell survival proteins like Bcl- x_L (M E Keir et al., 2008) (Figure 7). Although, stimulation of PD-1 outcome seems to be clear in T-cell function, the discovery of PD-L1 reverse signaling leaves a broader field to explore. Binding of PD-L1 has been proved to inhibit DCs activation and production of IL-10 (Kuipers et al., 2006). PD-L1 has been showed to be expressed in T cells, DCs, monocytes, lung, vascular endothelium, liver, keratinocytes and placental synctiotrophoblasts, however, there is a lot of data missing from other tissues (M E Keir et al., 2008). Since there is no published data for the expression of PD-L1 in MKs or platelets, it is relevant to search for its expression and possible role in pathogenesis.

I.6 HLA-DR expression in ITP

Expression of HLA-DR in HSCs has been clearly shown in previous studies. However, changes of HLA-DR expression across MK differentiation lacks more detailed description. Previous studies showed persistence of HLA-DR expression in megakaryoblasts in patients with increased proliferation of this cell lineage. The high heterogeneity of surface markers profile was associated to different stages of differentiation and suggested that HLA-DR can be expressed until megakaryoblasts phase

(Dent & Berkowitz, 1987). Nevertheless, results from other experiments would propose the total loss of MHC class II expression at earlier stages (Tomer, 2004).

In the case of ITP, data is quite limited and sometimes contradictory. Gaiger et al. studied the role of HLA-DR and –DP in adult thrombocytopenia, finding no association (Gaiger et al., 1994). On the other hand, Semple et al. reported increased expression of HLA-DR in ITP patients, without linking it directly to the disease pathogenesis, but considering an effect of a subjacent cause (John W. Semple & Freedman, 1995). Nevertheless, further data is required to establish the real role of HLA-DR abnormalities in ITP.

My research will focus on establishing a model of megakaryopoiesis and platelet development. Initial studies will explore the expression of PDL-1 and MHC class II over time to add to the literature of surface expression and to define for the first time the potential for interactions of T cells with MKs and platelets. Second, the impact of healthy and ITP plasma on megakaryopoiesis and platelet production and expression of immune surface markers will be explored.

I.7 Hypothesis and aims

Hypothesis

Megakaryopoiesis is compromised in patients with ITP, contributing to thrombocytopenia. Development of a model of megakaryopoiesis will allow examination of the potential for MKs to interact with immune cells and allow exploration of the impact of healthy and patient plasma on these features.

General aim

To determine whether plasma of ITP patients alters characteristics of megakaryopoiesis including terminal development and expression of immune markers including PD-L1 and HLA-DR.

Specific aims

1. To provide an experimental model to evaluate megakaryopoiesis by differentiating $CD34^+$ HSC into MKs.

2. To establish for the first time expression of PDL-1 and HLA-DR over time in megakaryopoiesis in standard media conditions.
3. To describe the effect of ITP plasma on MK development and immune surface make

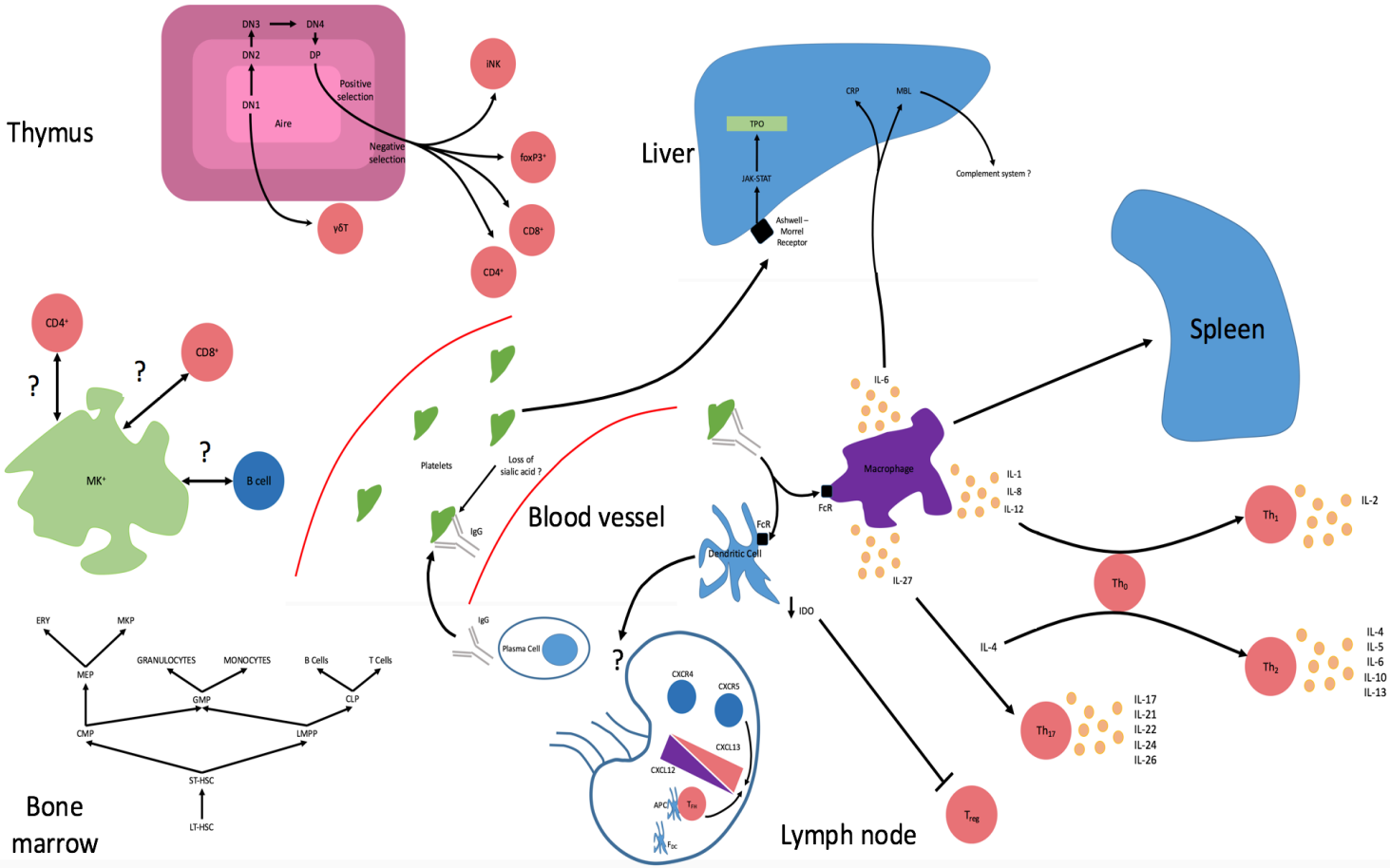


Figure 6. Current knowledge of ITP pathogenesis. Scientific literature presents multiple gaps in ITP's pathogenesis. Currently there is no accepted aetiology or hypothetic model. Nevertheless, compromise of central and peripheral tolerance has been proven, although the mechanisms behind them are still a mystery. Thymus involvement is still to be studied, nevertheless, self-reactivity of T cells certainly implies a maturation disorder in this subset of immune cells. The dynamics of T cells and B cells in bone marrow is also a subject without proper understanding. Whether there is a developmental failure in MKs is also a matter that has not been studied in detailed.
For many years it has been known that there is a loss of tolerance that triggers in most ITP patients' production of antibodies against platelets' surface markers. These antibodies start a predictable sequence of immune events that involve endocytosis of platelets and activation of macrophages, production of cytokines and maturation of different subsets of T lymphocytes. There is contradictory data about the cytokines involved in ITP, but it is generally accepted that it has a tendency for a Th_1 profile.

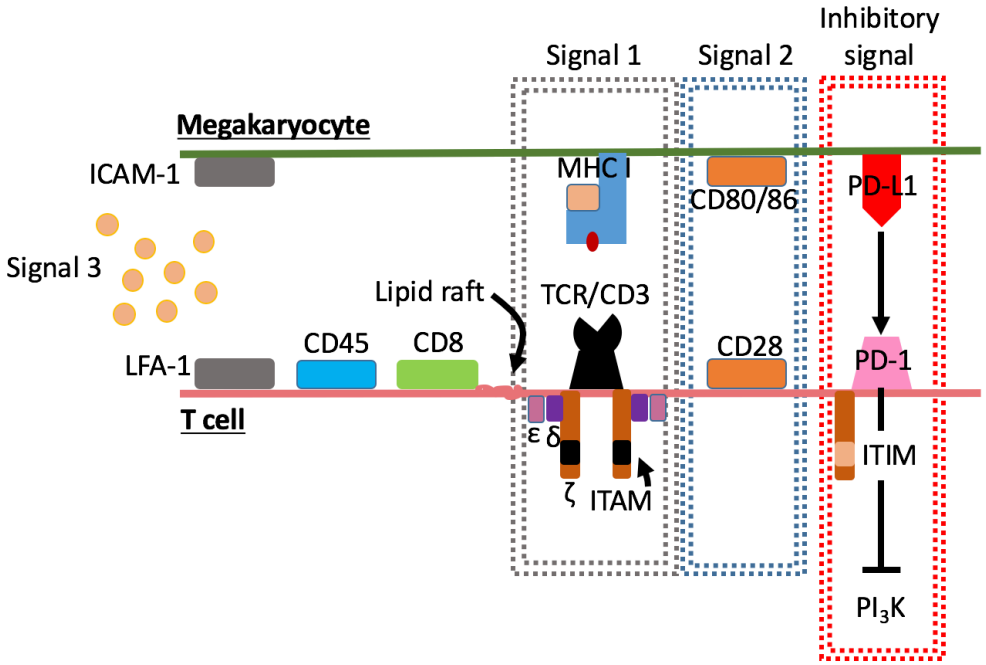


Figure 7. Model of immunological synapse between MKs and T cells. Three activation signals are required for T cells activation. These triad is composed by the binding of MHC molecule with TCR, CD80/86 with CD28 and the cytokines stimuli. In the absence of a strong MHC signal, cell inhibition can be induced by stimulation of PD-1 by PD-L1, which blocks PI_3K and further cytokines production.

Chapter II. Materials and method

II.1 Cells and culture

II.1.1 HSC cells

HSC Cells were obtained from Hammersmith Hospital's Stem Cell bank. $CD34^+$ cells were separated from the bone marrow of a healthy donor using magnetic beads. Cells were counted using a hemocytometer (Marienfeld, Germany) and stored in (10% Dimethyl Sulfoxide ((DMSO; Sigma-Aldrich, UK) + 90% Foetal Bovine Serum (FBS)) at a concentration of 12 x 10^6 cell/mL and stored in the -80°C freezer in the Centre for Haematology at Imperial College London, Hammersmith Campus. For thawing and revitalisation of cells, vials were opened and 1mL of warm PBS was added into the vial and thawed content was taken into a 15mL falcon tube with 10mL of PBS. Cells were centrifuged at 1600rpm for 5min, supernatant eliminated and re-suspended in 10mL of PBS to repeat a second wash and, then re-suspended in culture medium.

II.1.2 Medium

Cells were cultured in StemSpan™ SFEM II (STEMCELL™ Technologies, Cambridge, UK), and supplemented with StemSpan™ Megakaryocyte expansion supplement (STEMCELL™ Technologies), according to manufacturers' instructions. Additionally, TPO (Fisher Scientific, Loughborough, UK) was added at a concentration of 50ng/mL and 1µL of IL-1β (Fisher Scientific). Medium was filtered with a 0.2µm pore syringe filter to prevent any possible contamination. Cells were cultured at a concentration of 5 x 10^5 cells per mL in a 50mL flask and incubated at 37°C and 5% CO_2. Culture medium was renewed at day 5 and 7.

II.1.3 Plate seeding

At day 7, 3 x 10^5 cells per well in 24 wells plates. Cells were we-suspended in 400µL of medium and 100µL of plasma according to established condition (Figure 8). Cells cultured in medium only were re-suspended in 500µL. Remaining wells were filled with PBS to provide sufficient humidity. Cells were cultured at 37°C and 5% CO_2 for

additional three days before the they were harvested and analysed.

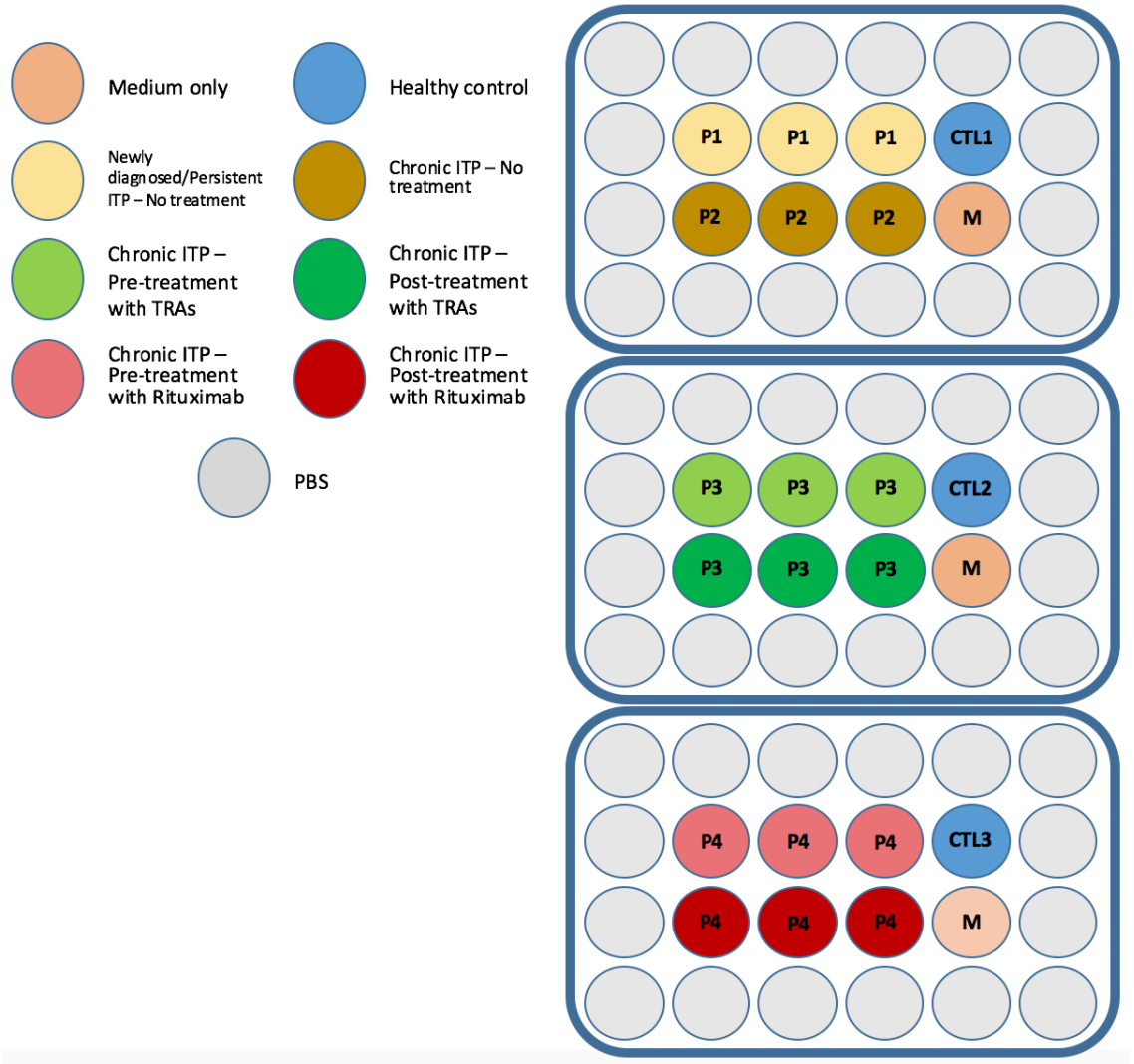


Figure 8. Cells plating and conditions. The three plates show the distribution of different conditions of culture. The **top plate** shows the cells cultured with plasma of 2 patients, one newly diagnosed, and the other a chronic patient. In both cases none of them were receiving medication. In the **middle plate**, cells were exposed to plasma of a chronic patient at two different time points: before starting treatment with TRAs and after showing platelets count improvement. The **bottom plate**, shows the plating of cells for testing plasma of a chronic ITP patient before and after treatment with Rituximab. Finally, in every plate, 1 well was used to test plasma of a healthy donor and another well for medium only control.

II.2 Patients' characterisation and Ethics

Patients' characterisation

4 patients with diagnosis of ITP, which are being followed at the Haematology service of Hammersmith's Hospital, were selected. Figure 9.

The criteria used for the selection of patients was based in 3 specific points:

1. Availability of sample.
2. Good quality of plasma.
3. Response to treatment shown by increased platelets.

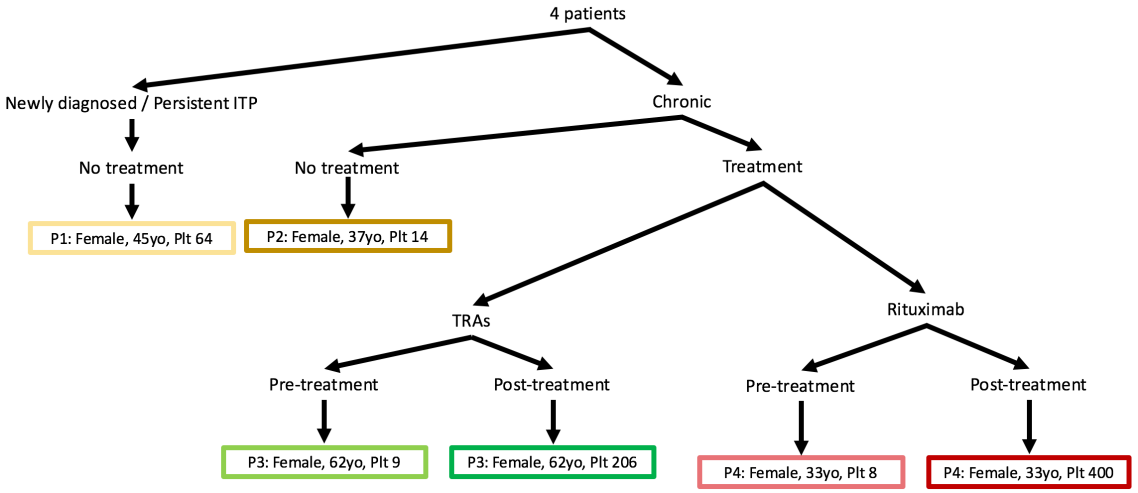


Figure 9. Patients characteristics. The figure shows some characteristics of the selected patients according to diagnosis, and treatment. Additionally, it provides some demographic information as gender, age, and platelets count at the time the sample was taken.

Ethics

Imperial College Tissue Bank granted approval to the study (REC Wales approval: 12/WA/0196 R12033). Tissue bank consent form was applied to get written informed consent from participants.

II.3 Flow cytometric analysis

II.3.1 Sample staining and flow cytometry analysis

The selection of fluorochromes was determined in light of the commercially available options and instrument configuration; lasers and detectors. A multi-colour panel of 8 primary conjugated antibodies (as defined in Table 2) was used to study MK differentiation process; CD3 (dump channel), CD34, CD38, CD41, CD42b, CD274 and HLA-DR. Live cell discrimination was achieved using Live/Dead Fixable Aqua (Fisher Scientific).

Live/Dead (Fisher Scientific) staining with performed by adding 0.5µL per sample, including both Fluorescence Minus One (FMO) controls and fully stained cells, diluted in 1 ml of PBS. Cells were incubated for 20 to 30min in the dark at 4°C and then washed and re-suspended with 100µL of PBS for Fc blocking.

For Fc blocking, to prevent non-specific binding, 2.5μL of Human Fc Block (BD Bioscience) was added to re-suspended cells and incubated for 10min in the dark at room temperature. One further wash in PBS was performed at 1600 rpm for 5min.

To stain, roughly 3 x 10^5 cells were prepared in 100 μL of PBS in separate FACS tubes. A master mix of antibody cocktail was calculated so that each sample is stained with, 1μL of anti-CD3, anti-CD34, anti-CD38, anti-CD41, anti-CD42b, antiHLA-DR and 2μL of anti-CD274. Samples were incubated with the master mix in the dark at 4°C for 20min. Then, cells were washed and re-suspended in 400μL of PBS ready for analysis.

Unstained control, FMO controls were included with each experimental run to allow for compensation and determine negative quadrants, respectively. To compensate for potential spectral overlap between the neighbouring channels, single stained compensation beads BD™ CompBeads Set (BD Bioscience, USA) was included for each antibody with the exception of the L/D single stained control that was prepared by mixing cooled killed cells (by boiling in a water bath) with unstained live cells and stained with LD reagent as detailed above.

Before acquisition, PMT values were adjusted using unstained control to restrain the negative population typically below 10^2 log. Single-stained controls were then acquired at 5000 events (by default) per tube. The automated compensation matrix of acquired control tubes was computed using FACSDiva; no post acquisition compensation was required.

Data acquisition was undertaken on FACSAria II (BD Bioscience) with 3 lasers system (Violet 407 nm, Blue 488 nm and Red 633nm) in the BRC core facility laboratory housed at the Common Wealth Building, Imperial College London. Table 4 shows the settings for the channels' filters established for fluorescence detection.

Data analysis was subsequently performed using FlowJo v. 8.7, Treestar flow cytometry analysis software.

II.4 Confocal Microscopy

To detect the surface expression of PD-L1 at day 3, day 7 and day 10, slides were prepared with cytospinning, stained cells on glass slides and mount with Fluoroshield™ with 4',6-diamidino-2-phenylindole (DAPI; (Sigma-Aldrich, USA), and visualised using an inverted Zeiss LSM-780 confocal microscope (Zeiss, Oberkochen, Germany), and Zen Lite Software (Zeiss). Further, analysis and edition was fulfilled with FIJI (Schindelin et al., 2012)

Table 2. Antibodies and reagents used for staining

Antibody	Description	Fluorochrome	Company
CD3	Mouse Anti-Human	V450	BD Bioscience
CD34	Mouse Anti-Human	PECy7	eBioscience, UK
CD38	Mouse Anti-Human	AlexaFluor 700	Biolegend, UK
CD41	Mouse Anti-Human	APC	Biolegend
CD42b	Mouse Anti-Human	PE	eBioscience
CD274	Mouse Anti-Human	FITC	AbD Serotec
HLA-DR	Mouse Anti-Human	PerCP	Biolegend
Live/Dead		Fixable Aqua	Fisher Scientific

II.5 Statistical analysis

All data are expressed as mean ± SD unless indicated otherwise. Statistical comparison between two groups was carried out using unpaired *t* tests, using Prism 7.0 for MacOS X (GraphPad Software Inc, USA). Results were considered statistically significant when $p<0.05$.

Table 3. FACSAria II Filters according to specific fluorochrome

Fluorochrome	Filter
V450	450/40V
Fixable Aqua (L/D)	525/50V
FITC	525/50
PE	575/25
PerCP	670/14
PECy7	780/60
APC	670/14R
AlexaFluor 700	710/50R

Chapter III. Results

III.1 Gating strategy

Gating strategy for flow cytometry analysis was established as shown in Figure 10. Side-light- Scattered SSC-A) vs Forward-light-Scattered (FSC-A) was used to gate the population of interest. The exclusion of dead cells and elimination of doublets was performed by first gating out L/D^{+} cell population and then FSC-W high cells on serial bivariate dot plots (LD vs FSC-A) and then (FSC-W vs FSC-A), respectively. Finally, we proceeded to exclude cells expressing surface CD3.

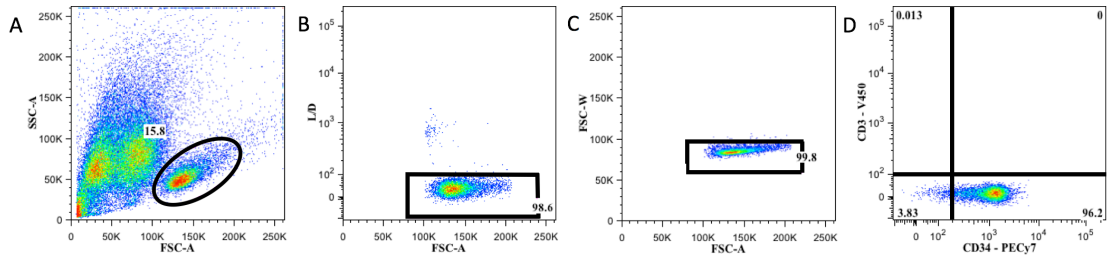


Figure 10. Gating strategy for developing MKs. A. FSC-A vs SSC-A. **B.** Gating for alive cells. **C.** FSC-A vs FSC-W, gating of single cells. **D.** CD34 – PECy7 vs CD3 – V450 for exclusion of cells expressing CD3 in its surface.

III.2 Effect of post staining cell fixation on PE signal

Cell fixation with CellFIX (BD Bioscience, UK) post sample staining used normally to allow for later acquisition led to a significant upward displacement of PE negative population of PE negative control (PE-FMO) causing an overlap with and loss of PE positive events in the fully stained samples (Figure 11). This effect was not observed on any of the other fluorochromes used. Fresh, unfixed, cells showed the expected separation between PE, PE-FMO and fully stained tube containing PE+ cell populations (Figure 11). It has been therefore decided to run all samples freshly within 24 hours of sample staining.

III.3 Optimization of negative controls

Unstained control with FMO for PerCP, FITC and PE were compared to determine the optimal negative controls to accurately discriminate positive versus

negative signals and draw gates. Whilst there was no signal difference between unstained control, FITC-FMO and PE-FMO, PerCP-FMO showed considerable positive signal shift of PerCP- population. As it is shown in (Figure 12 B), PerCP FMO was more representative of negative controls than unstained control. PerCP- FMO was therefore used to determine PerCP negative and positive events in subsequent analysis.

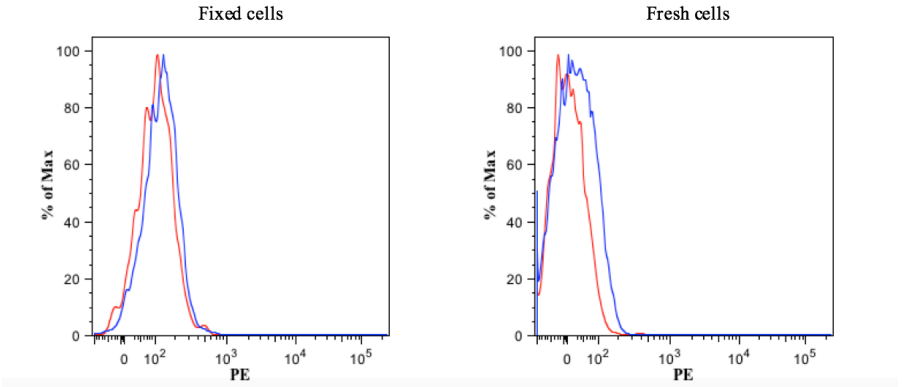


Figure 11. Fixation of cells alters fluorescence profile. Analysis PE-FMO (red) and fully stained (blue) MKP on day 7.

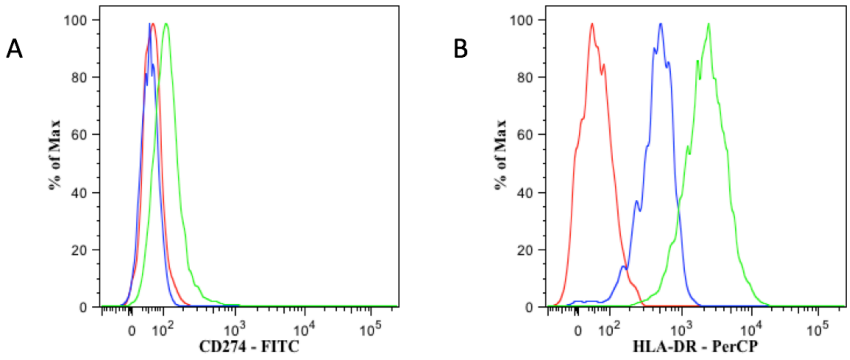


Figure 12. Fluorescence for FITC and PerCP in unstained (red), FMO (blue) and fully stained (green) cells. MKP on day 3 stained with full panel. **A.** Displays of fluorescence detected by FITC channel. **B.** Displays the fluorescence detected by the HLA-DR channel.

III.4 MK culture and development

The outcome of MKs' culture optimisation showed appropriate production of mature MKs. Using MK culture procedures explained in material and methods section II.1.2. Cultured $CD34^+$ HSC started to express one of the earlier MK marker CD41, continuing to increase significantly throughout MK development from around 18% on day 3 reaching over 90% on day 10. CD42b expression starts to appear as early as day 3 and continues to rise up as its dual expression with CD41 is a marker of mature MK (Figure 14A).

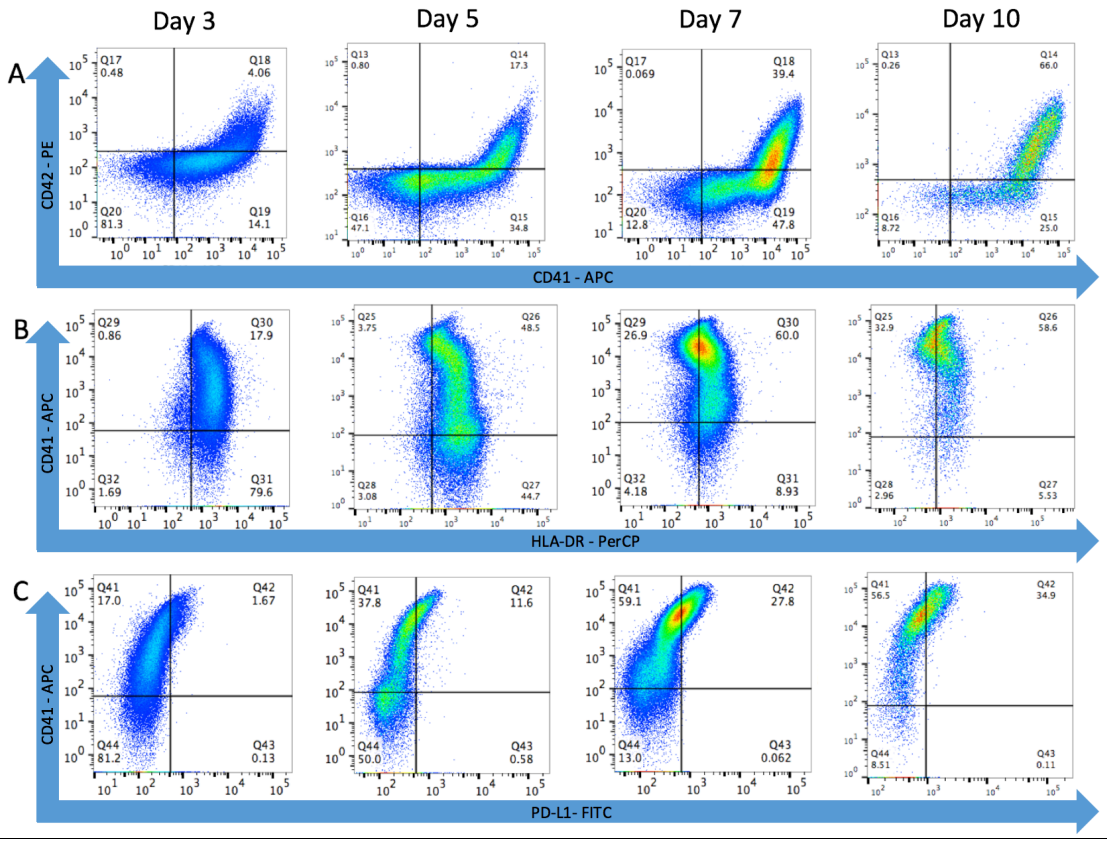


Figure 14. MK development and 10 days. A. Displays the increasing expression of CD4 and CD42b over time, showing displacement of the whole population towards a double positivity which is a marker of mature MK. **B.** Displays the novel finding of HLA-DR expression during the course of MK development, overall decrease across the developing cells. **C.** Shows PD-L1 expression in relation to CD41which shows increase through MK differentiation. Images show $CD41^{+}$ preceding the expression of PD-L1.

Early progenitor cells highly express HLA-DR, which sums up to over 97% on day 3. Following the development of the cells a significant reduction is observed throughout the culture ending around 63% on day 10 (Figure 14B). The expression of the inhibitory marker PD-L1 seemed to be exclusive to $CD41^{+}$ cells and also to follow its progression as it increases through the development of MK from 11% on day 5 to 35% on day 10 (Figure 14C).

III.5 MK development and expression of surface markers culture in replicates

Culture replicates at day 10 showed expression of CD41 in medium reached a mean average of 13.19% (SD±6.83). $CD42b^{+}CD41^{+}$ cells only represented 0.27% (SD±0.23). Loss of CD34 expression reached 11.42% (SD±7.99) (Figure 15).

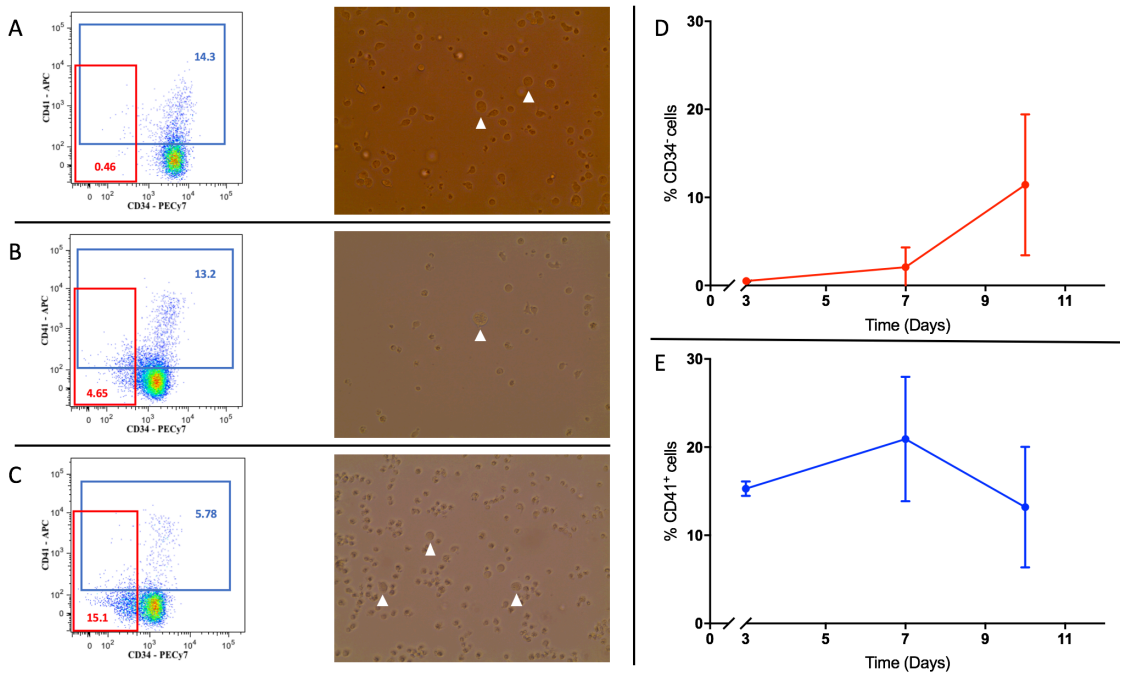


Figure 15. Number of cells (medium only) expressing CD34 and CD41 across development. A. Day 3, B. Day 7, C. Day 10. Dot plot showing expression of CD34 and CD41 (left). Photographs (40X) of cells showing phenotypic changes (white arrows). (right). **D.** Line graph showing loss of expression of CD34. **E.** Line graph showing variation in cell number expressing CD41.

III.6 MK differentiation is enhanced in the presence of healthy control and ITP plasma

Cells exposed to plasma (Healthy control and ITP) showed an increased the overall expression of CD41 as well the percentage of the terminally differentiated $CD41^{+}CD42b^{+}CD38^{+}$ cells.

Expression of CD41 with plasma of a healthy donor ascended to 45.98% (SD±10.23) and with plasma from ITP patients to 28.29% (SD±13.94), compared to 13.19% in medium only. The development of mature MK cells increased significantly to 36.1% (SD±7.40) in the presence of ITP plasma compared to medium only, 14.3% (P value <0.05). Healthy donor's plasma also had a similar increasing effect but it did not reach a statistical significance when compared to medium only (Figure 16).

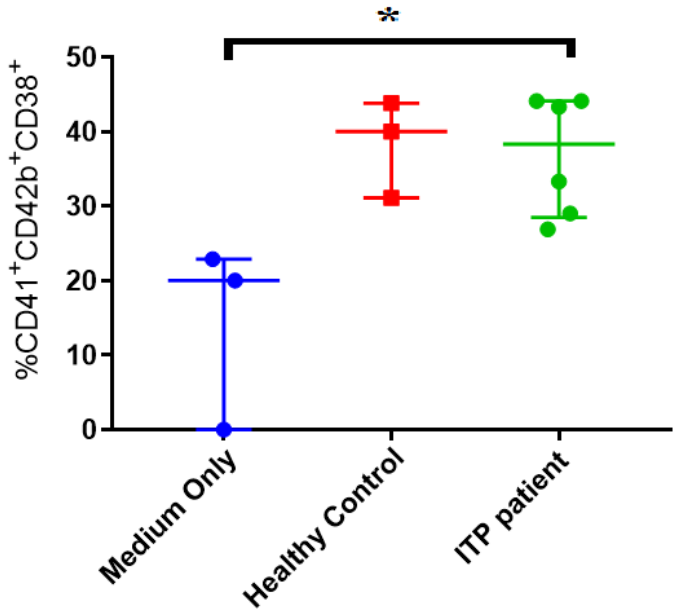


Figure 16. CD41^{+}CD42b^{+}CD38^{+}MK maturation cultured in medium only, plasma of a healthy control or ITP patients. The graph shows an overall increase when cultured with plasma from either a healthy donor or an ITP patient compared to medium only. It also shows a significant increase in the development of mature MK when cultured with ITP plasma compared to medium only. * P value <0.05

III.7 HLA expression is maintained in the presence of ITP patient plasma

During MK development, the expression of HLA-DR was found to be decreasing as the cells differentiate when left in medium only. Culturing the cells with plasma from a healthy donor further reduced the expression of HLA-DR significantly to 53.2% when compared to ITP plasma at 75.4%. The difference in HLA-DR expression was not of statistical significance between the cells cultured in medium only and with ITP plasma, showing positivity in 81.23% and 75.4% of the cells, respectively (Figure 17).

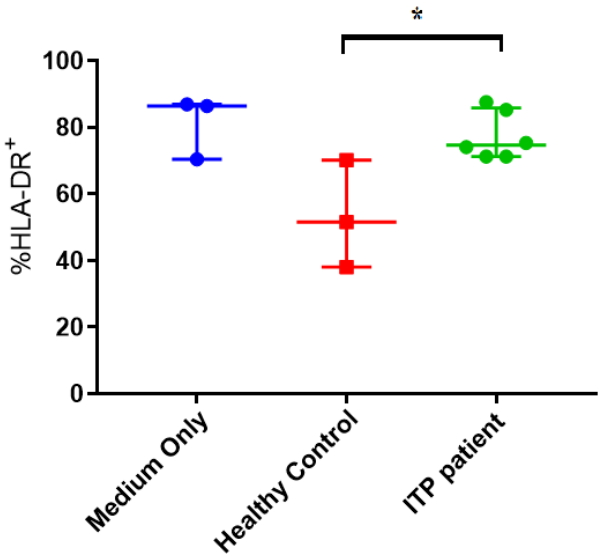


Figure 17. HLA expression of cells cultured in medium only, plasma of a healthy control or ITP patients. The graph shows an overall decrease when cultured with plasma from either a healthy donor or an ITP patient compared to medium only. Major reduction in HLA-DR expression is achieved with healthy plasma compared to ITP plasma. * P value <0.05

III.8 PD-L1 expression is increased in the presence of healthy control and ITP patient plasma

Expression of PD-L1 seemed to be increasing through the development of MK reaching 21.13% (SD±16.99) after 10 days in culture medium. An observed rise in the expression of PD-L1 when cultured with healthy control plasma and ITP plasma to 25.06% (SD±19.10) and 17.21% (SD±8.26) respectively (Figure 18)(Figure 19).

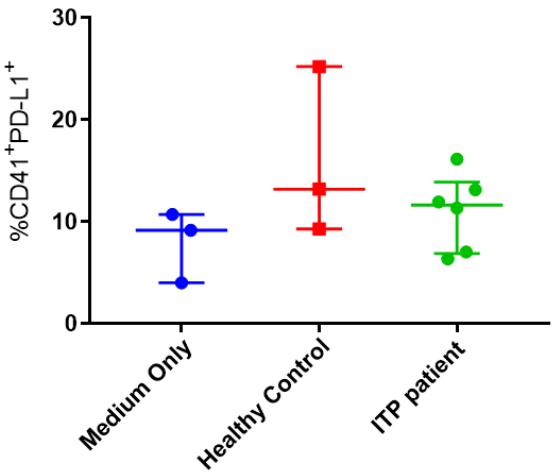


Figure 18. Expression PD-L1 in cells cultured in medium only, plasma of a healthy control or ITP patients. The graph demonstrates an overall increase in PD-L1 expression when cultured with plasma from either a healthy donor or an ITP patient for three days compared to medium only. No significant difference in expression was observed when cells were cultured with either healthy controls or ITP plasma.

The surface expression of PD-L1 on mature MK was confirmed using confocal microscopy which showed distinctive surface expression that differed from both CD41 and CD42b receptors (Figure 18).

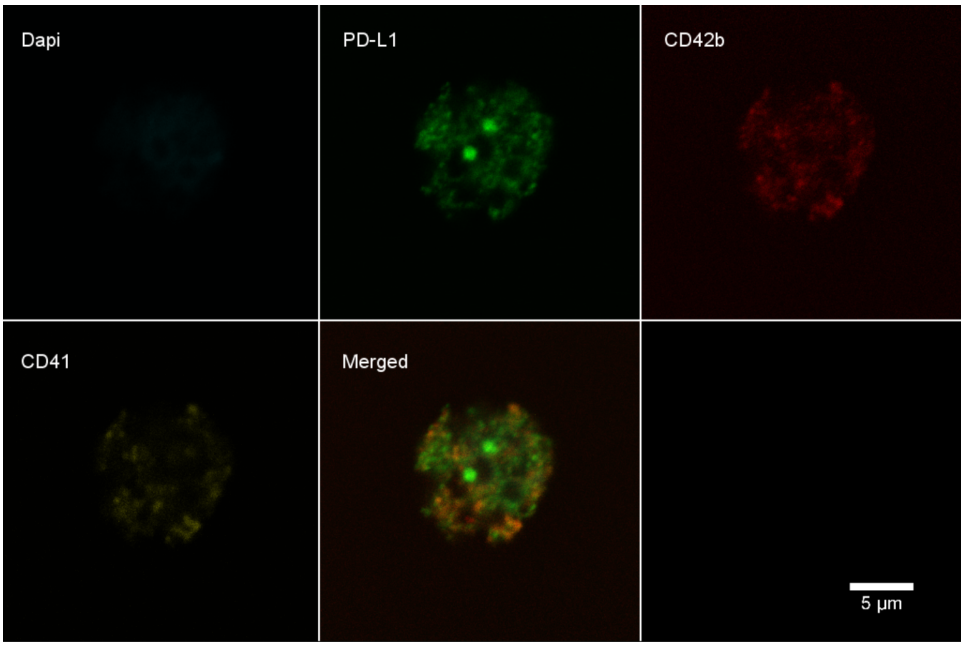


Figure 19. PD-L1 expression in mature MK. A single mature MK stained captured with different channels for each fluorochrome; DAPI (Blue); PD-L1 (Green); CD42b (Red) and CD41 (Yellow). Lower middle image represents when all the channels are combined. The distinctive expression of PD-L1 can be appreciated in the merged channel where green colour stands by itself not overlapping with any other channel, as in the case with CD41 and CD42b appearing orange in some areas in the merged channel. Image was captured using an oil lens with X63 magnification.

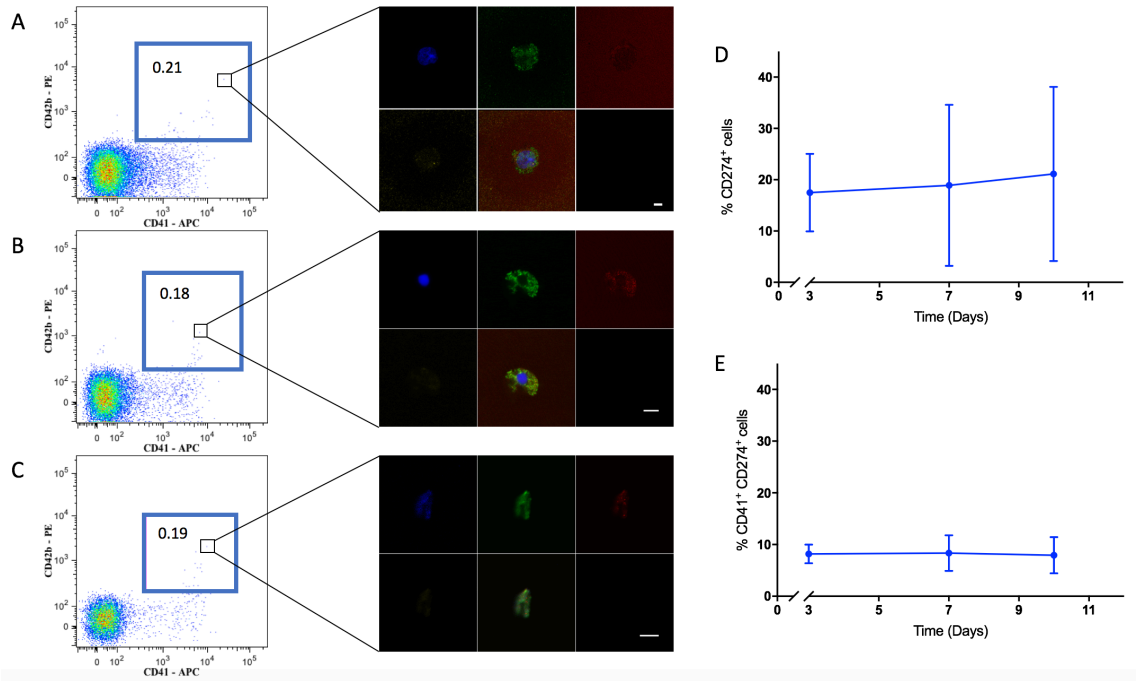


Figure 20. Expression of PD-L1 in MKs. A. Day 3, **B.** Day 7, **C.** Day 10. Dot plot for CD41 vs CD42b (left). Confocal microscopy (right) of stained cells with DAPI (Blue), PD-L1 (Green), CD42b (Red) and CD41 (Yellow). Lower middle image represents the merged image. Lower right image shows scale for 5µm. **D.** Line graph showing average expression of PD-L1. **E.** Line graph showing expression of double positive cells for CD41 and PD-L1.

Cells were cultured and analysed in ITP plasma of patients post-treatment once for Rituximab and TRAs. Expression of surface markers after rituximab treatment showed 17.3% of $CD34^-$, 55.8% of $CD41^+$ cells, 6.58% of $CD42b^+CD41^+$, 26.9% of $CD42b^+CD41^+CD38^+$, 75.3% of $HLA\text{-}DR^+$, 18% of $CD41^+$ $HLA\text{-}DR^-$, 8.59% of $PD\text{-}L1^+$ and 6.35% of $CD41^+PD\text{-}L1^+$.

Post TRA treatment plasma presented 6.58% of $CD34^-$, 25.2% of $CD41^+$ cells, 8.44% of $CD42b^+CD41^+$, 44.1% of $CD42b^+CD41^+CD38^+$, 87.5% of $HLA\text{-}DR^+$, 4.43% of $CD41^+$ $HLA\text{-}DR^-$, 17.5% of $PD\text{-}L1^+$ and 13.1% of $CD41^+PD\text{-}L1^+$.

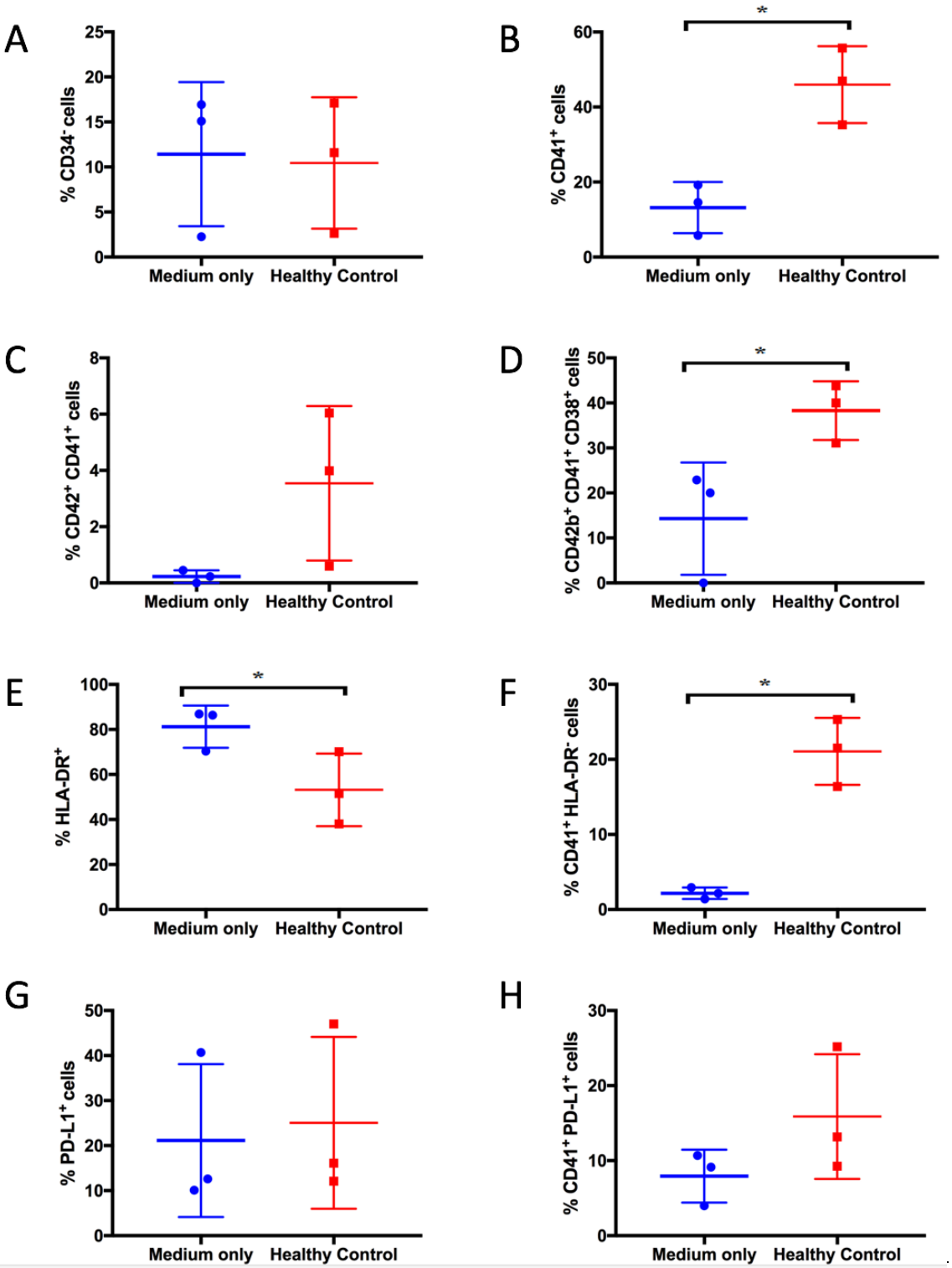


Figure 21. Contrast of surface markers expression between cells cultured in medium only and with healthy control plasma. Unpaired T-test analysis. Data shown from three different cultures after 10 days of differentiation. **A.** Percentage of cells expressing CD34. **B.** Percentage of cells expressing CD41 ($p<0.05$). **C.** Percentage of cells $CD42b^{+}CD41^{+}$. **D.** Percentage of cells $CD42b^{+}CD41^{+}CD38^{+}$ ($p<0.05$). **E.** Percentage of cells expressing HLA-DR ($p<0.05$). **F.** Percentage of cells $CD41^{+}HLA\text{-}DR^{-}$ ($p<0.05$). **G.** Percentage of cells expressing PD-L1. **H.** Percentage of cells $CD41^{+}PD\text{-}L1^{+}$.

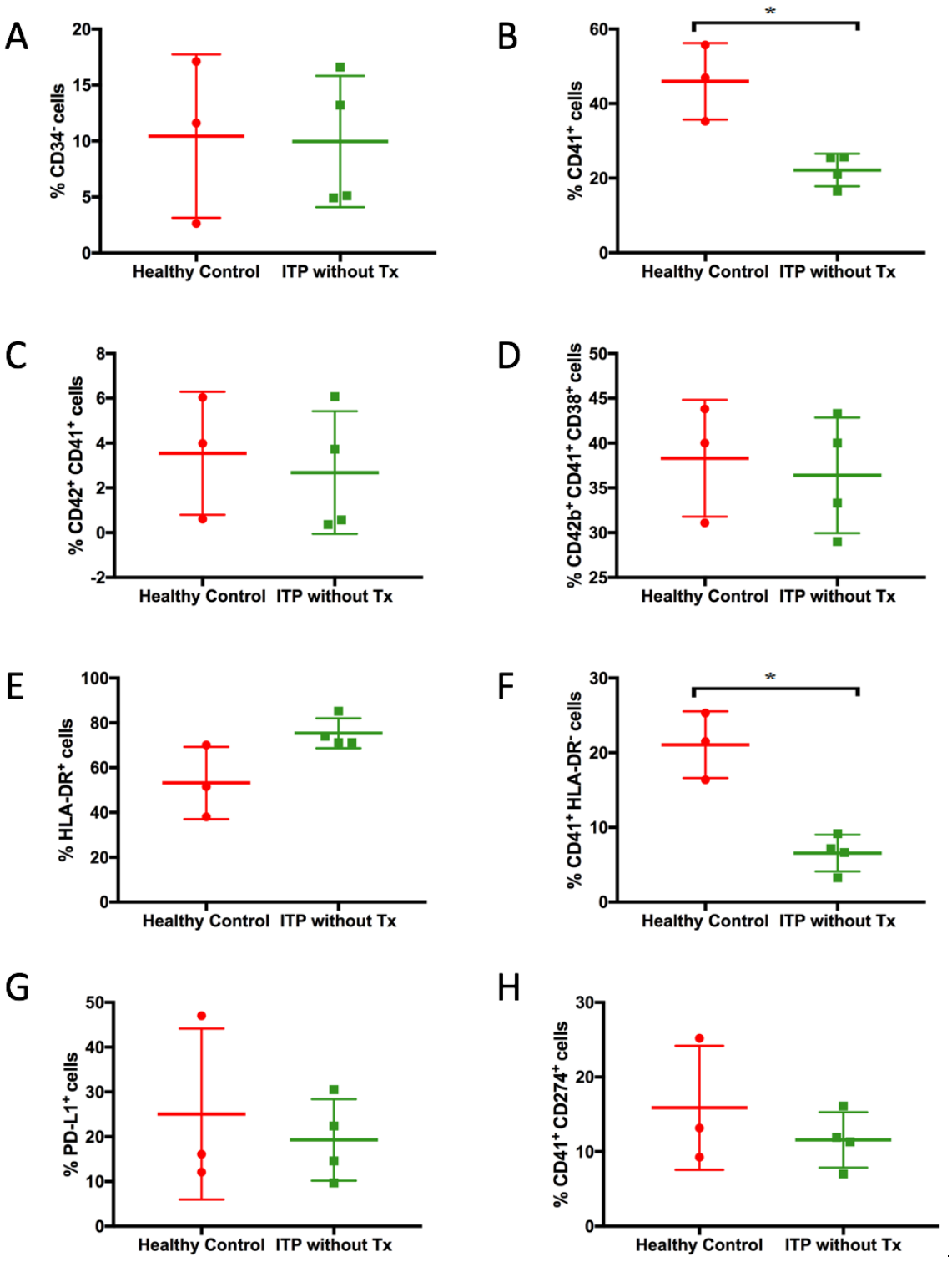


Figure 22. Contrast of surface markers expression between cells cultured with healthy control plasma and ITP plasma without treatment. Unpaired T-test analysis. Data shown from three different cultures after 10 days of differentiation. **A.** Percentage of cells expressing CD34. **B.** Percentage of cells expressing CD41 ($p<0.01$). **C.** Percentage of cells $CD42b^{+}CD41^{+}$. **D.** Percentage of cells $CD42b^{+}CD41^{+}CD38^{+}$. **E.** Percentage of cells expressing HLA-DR. **F.** Percentage of cells $CD41^{+}HLA\text{-}DR^{-}$ ($p<0.01$). **G.** Percentage of cells expressing PD-L1. **H.** Percentage of cells $CD41^{+}PD\text{-}L1^{+}$.

Chapter IV. Discussion

Megakaryocytes (MKs) derive from haematopoietic stem cells (HSCs) by a process called megakaryopoiesis (Deutsch & Tomer, 2006). Once a lineage commitment has been established, development towards mature MKs will be characterised by DNA endoreduplication, cytoplasmic expansion and release of cytoplasmic fragments or platelets (Deutsch & Tomer, 2006). The different stages in the development of MKs can be described in the following sequence: burst-forming units MKs (BFU-MK), Colony forming units MKs (CFU-MK), pro-megakaryoblasts, megakaryoblasts and MKs. The evolution across these stages are based in the development of polyploidy, which gets up to 128n (Ravid, Lu, Zimmet, & Jones, 2002). For the recognition of these stages, the surface expression of glycoproteins is considered a useful tool (Tomer, 2004).

The purpose of these differentiated cells is to produce platelets, which are involved in the development of blood clots and wound healing. Immune thrombocytopenia (ITP) is defined as a reduction in the platelet count in the absence of a clear cause (Kistangari & McCrae, 2013). Although traditionally thought to result from increased platelet destruction, impaired megakaryopoiesis is also relevant in ITP. In order to understand the reasons for the trigger of the immune system against MKs and platelets, we need to know in detail the differentiation process. Although, there is information of MKs development in humans, most data about HSCs differentiation into different lineages has been the result of murine studies. Hence, data cannot necessarily be extrapolated to humans and further modelling of MKs development is required in humans.

We have demonstrated MK development in the laboratory. The culture protocol showed consistent production of $CD42b^{+}CD41^{+}$ cells. Also, the progression of surface markers expression in time, showed compatibility with the previous data published (Tomer, 2004). The efficiency of our protocol for MK differentiation showed some variability between different cultures and requires further optimisation. Nonetheless, lineage commitment to MK was solidly established. Since there was a recognisable increase in the amount of dead cells in latter cultures, efficiency in cell differentiation should require a careful study of stored HSCs viability. It has been demonstrated that cryopreservation keeps HSCs properties (Fleming & Hubel, 2006; Spurr, Wiggins,

Marsden, Lowenthal, & Ragg, 2002), but whether if specific subsets, like MK biased HSCs (Bryckaert, Rosa, Denis, & Lenting, 2015), are especially vulnerable or not to cryopreservation, is unknown.

The culture of MKs over time also showed a progressive decrease in the expression of HLA-DR over time, consistent with published data (REF). The continued presence of some HLA-DR positive cells may relate to the early termination of the culture (culture to day 12 and production of platelets may result in further decrease in HLA-DR).

The progression of HSCs towards MKS was enhanced with the addition of healthy plasma at day 7. There was a significant increase of $CD41^{+}$ cells and decrease in the expression of HLA-DR in cells cultured with healthy plasma. The addition of ITP plasma did not show higher expression of CD41+ cells compared to medium, but did show a preservation of HLA-DR expression. This resulted in a lower number of CD41+HLA-DR- cells in cells cultured with ITP plasma compared to those cultured with healthy plasma ($p<0.05$).

This outcome allows us to state that the addition of plasma at day 7 enhances the differentiation of MKs and that plasma can modulate not only the differentiation of MKs, but also the expression of surface markers which may result in a different role for these MKs. Previous studies suggest megakaryopoiesis is inhibited when cultured with ITP plasma, however plasma was added at day 0 in these cultures (Chang et al., 2003; Robert McMillan et al., 2004). Further work is clearly required.

The relatively lower CD41+ cells in cells cultured in patient plasma may reflect the presence of anti-platelet antibodies or other plasma derived factors. Some studies suggest that alterations in MK development are mainly associated to antibodies' activity (Psaila & Bussel, 2007). Nevertheless, 30% of ITP patients lack anti-platelet antibodies. It still has to be determined, whether these patients produce different, undiscovered antibodies, or if the cytokine profile induces alterations in MK development (Chanock, 2003).

In respect to HLA-DR expression, previous studies have not shown presence of HLA-DR in mature MK. Current literature states that HLA-DR expression is lost in early stages of MK development in fresh bone marrow samples (Tomer, 2004). This has not

been studied in vitro culture expansion. In our experiment, we showed that healthy plasma significantly decreases the expression of HLA-DR compared to ITP plasma. These results support a case study that suggests that cytokines in plasma induce HLA-DR expression in platelets in patients with ITP (Boshkov, Kelton, & Halloran, 1992). The role of HLA-DR in ITP pathogenesis has not been settled. Some studies could not link HLA phenotype to disease risk and evolution (Gaiger et al., 1994). Whether the phenotypic changes in MKs seen with plasma perpetuates the disease or is a consequence of the disease needs to be determined.

Although no statistical difference has been proven in the expression of PD-L1, its presence has been persistent in several time points, and seems that it is not affected by the differentiation process. Analysis of confocal microscopy images confirms the presence of PD-L1 in $CD42b^{+}CD41^{+}$ cells. More studies are needed with an optimised culture, but the regularity of PD-L1 expression, even in cells with control and ITP plasma, allow us to state that we have shown for the first time the expression of PD-L1 in MKs (M E Keir et al., 2008)(Chen & Han, 2015).

No significant differences were obtained with other markers. However, an optimised culture should provide a bigger sample for analysis, and additional differences cannot be discarded. Nonetheless, we consider that the demonstration of differences between healthy control and ITP patients without treatment is a good starting point for further research.

To establish the clinical relevance of PD-L1 in ITP more studies will have to be carried out. It has been demonstrated that PD-L1 expression is altered by IFN-γ, GM-CSF, IL-4, IL-12 in multiple cells (Okazaki & Honjo, 2007), which would be relevant to ITP pathogenesis. Furthermore, polymorphisms of PD-1 receptor showed association with autoimmune diseases such as systemic lupus erythematous (SLE) and multiple sclerosis (MS) (M E Keir et al., 2008; Prokunina et al., 2002). Future work should focus on the confirmation of HLA-DR and PD-L1 *in vivo,* which would broaden the view of immune regulation with MK as a protagonist.

The tasks developed by MKs can be summarized in the production of two specific items: platelets and vWF. Platelets are enucleated structures and owe their properties to

MKs. Thus, it has been established that the role of MKs lies in the indirect maintenance of tissues and haemostatic regulation. Likewise, recent studies, have shown some evidence suggesting a significant role of platelets in host's defence against infections and inflammation (Beaulieu & Freedman, 2010; Duerschmied, Bode, & Ahrens, 2014; Z. Li, Yang, Dunn, Gross, & Smyth, 2011). The presence of CD40 ligand (CD40L), TLRs and pro-inflammatory and bactericidal molecules like α - granules, dense granules, lysosomes and defensins, are proof of platelet's potential in host defense (John W Semple, Italiano, & Freedman, 2011). Furthermore, studies in human diseases like parasitic infections (Mcmorran et al., 2009), sepsis (Z. Li et al., 2011), atherosclerosis, metabolic syndrome, asthma and ischemia/reperfusion syndrome, have demonstrated a wide response versatility, suggesting a higher role than commonly credited. Unfortunately, detailed understanding of these functions are still a work in progress (Duerschmied et al., 2014; John W Semple et al., 2011). Therefore, we venture to suggest that MK abnormalities translate into immunological disturbances that might not necessarily be clinically evident.

Further work will include standardisation of MK culture. Addition of plasma from day 0 might allow better differentiation and could show a more differences between conditions since cells would be exposed for a longer period of time to distinct cytokines. Also, plasma might induce differentiation of other cell lineages giving a more realistic depiction of cells development. Additionally, it will be necessary to discriminate effect of plasma cytokines from the effect that antibodies might be inducing.

Finally, we can conclude that MKP and mature MK can express PD-L1 *in vitro* culture. Also, healthy plasma and ITP plasma has the potential to enhance the differentiation process, as seen by the increased number of mature MK subsets. Cells cultured with ITP plasma will express less $CD41^+$ and $CD41^+HLA\text{-}DR^-$ cells than healthy controls. The possibility of immune regulatory properties require further investigation.

Chapter V. References

Adolfsson, J., Borge, O. J., Bryder, D., Theilgaard-Mönch, K., Åstrand-Grundström, I., Sitnicka, E., Sitnicka, E., Sasaki., Y & Jacobsen, S. E. . (2001). Upregulation of Flt3 Expression within the Bone Marrow Lin−Sca1+c-kit+ Stem Cell Compartment Is Accompanied by Loss of Self-Renewal Capacity. *Immunity*, *15*(4), 659–669.

Adolfsson, J., Månsson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C. T., Bryder, D., Yang, L., Borge, O., Thoren, Lina A., Anderson, K., Sitnicka, E., Sasaki, Y., Sigvardsson & M., Jacobsen, S. E. W. (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential: A revised road map for adult blood lineage commitment. *Cell*, *121*(2), 295–306.

Al-Chaqmaqchi, H., Sadeghi, B., Abedi-Valugerdi, M., Al-Hashmi, S., Fares, M., Kuiper, R., Lundahl, J., Hassan, M & Moshfegh, A. (2013). The Role of Programmed Cell Death Ligand-1 (PD-L1/CD274) in the Development of Graft versus Host Disease. *PLoS ONE*, *8*(4), 1–7.

Appay, V. (2004). The physiological role of cytotoxic CD4+ T-cells: The holy grail? *Clinical and Experimental Immunology*, *138*(1), 10–13.

Arnold, D. M., Dentali, F., Crowther, M. A., Meyer, R. M., Cook, R. J., Sigouin, C., & Kelton, J. G. (2007). Systematic review: Efficacy and safety of rituximab for adults with idiopathic thrombocytopenic purpura. *Annals of Internal Medicine*.

Attar, A. (2014). Changes in the Cell Surface Markers During Normal Hematopoiesis: A Guide to Cell Isolation. *Global Journal of Hematology and Blood Transfusion*, *1*, 20–28.

Beaulieu, L. M., & Freedman, J. E. (2010). The role of inflammation in regulating platelet production and function: Toll-like receptors in platelets and megakaryocytes. *Thrombosis Research*, *125*(3), 205–209.

Belyakov, I. M., Kozlowski, S., Mage, M., Ahlers, J. D., Boyd, L. F., Margulies, D. H., & Berzofsky, J. A. (2007). Role of a3 domain of class I MHC molecules in the activation

of high- and low-avidity CD8+ CTLs. *International Immunology*, *19*(12), 1413–1420.

Boshkov, L. K., Kelton, J. G., & Halloran, P. F. (1992). HLA-DR expression by platelets in acute idiopathic thrombocytopenic purpura. *British Journal of Haematology*, *81*(4), 552–557.

Bryckaert, M., Rosa, J.-P., Denis, C. V, & Lenting, P. J. (2015). Of von Willebrand factor and platelets. *Cellular and Molecular Life Sciences : CMLS*, *72*(2), 307–26.

Catani, L., Sollazzo, D., Trabanelli, S., Curti, A., Evangelisti, C., Polverelli, N., Palandri, F., Baccarani, M., Vianelli, N., & Lemoli, R. M. (2013). Decreased expression of indoleamine 2,3-dioxygenase 1 in dendritic cells contributes to impaired regulatory T cell development in immune thrombocytopenia. *Annals of Hematology*, *92*(1), 67–78.

Chang, M., Nakagawa, P. A., Williams, S. A., Schwartz, M. R., Imfeld, K. L., Buzby, J. S., & Nugent, D. J. (2003). Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro. *Blood*, *102*(3), 887–895.

Chanock, S. (2003). The etiology of childhood immune thrombocytopenic purpura: how complex is it? *Journal of Pediatric Hematology/Oncology*, *25 Suppl 1*(December), S7–10.

Chen, L., & Han, X. (2015). Anti – PD-1 / PD-L1 therapy of human cancer : past , present , and future. *The Journal of Clinical Investigation*, *125*(9), 3384–3391.

Cooper, N. (2009). Intravenous Immunoglobulin and Anti-RhD Therapy in the Management of Immune Thrombocytopenia. *Hematology/Oncology Clinics of North America*, *23*(6), 1317–1327.

Cooper, N., & Bussel, J. (2006). The pathogenesis of immune thrombocytopaenic purpura. *British Journal of Haematology*, *133*(4), 364–374.

D'Addio, F., Riella, L. V, Mfarrej, B. G., Chabtini, L., Adams, L. T., Yeung, M., Yagita, H., Azuma, M., Sayegh, M., & Guleria, I. (2011). The link between the PDL1

costimulatory pathway and Th17 in fetomaternal tolerance. *Journal of Immunology (Baltimore, Md. : 1950)*, *187*(9), 4530–41.

Dent, A., & Berkowitz, D. (1987). *Cell Surface Phenotyping of Megakaryoblasts*. *Blood* (Vol. 69).

Deutsch, V. R., & Tomer, A. (2006). Megakaryocyte development and platelet production. *British Journal of Haematology*, *134*(5), 453–466.

Draper, J. E., Sroczynska, P., Tsoulaki, O., Leong, H. S., Fadlullah, M. Z. H., Miller, C., Kouskoff, V., & Lacaud, G. (2016). RUNX1B Expression Is Highly Heterogeneous and Distinguishes Megakaryocytic and Erythroid Lineage Fate in Adult Mouse Hematopoiesis. *PLOS Genetics*, *12*(1), e1005814.

Duerschmied, D., Bode, C., & Ahrens, I. (2014). Immune functions of platelets. *Thrombosis and Haemostasis*, *112*(4), 678–691.

Emmerich, F., Bal, G., Barakat, A., Milz, J., Mühle, C., Martinez-Gamboa, L., Dörner, T., & Salama, A. (2007). High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura. *British Journal of Haematology*, *136*(2), 309–314.

Ertelt, J. M., Buyukbasaran, E. Z., Jiang, T. T., Rowe, J. H., Xin, L., & Way, S. S. (2013). B7-1/B7-2 blockade overrides the activation of protective CD8 T cells stimulated in the absence of Foxp3+ regulatory T cells. *Journal of Leukocyte Biology*, *94*(2), 367–76.

Eto, K., & Kunishima, S. (2016). Linkage between the mechanisms of thrombocytopenia and thrombopoiesis. *Blood*, *127*(10), 1234–1242.

Fleming, K. K., & Hubel, A. (2006). Cryopreservation of hematopoietic and non-hematopoietic stem cells. *Transfusion and Apheresis Science*, *34*(3), 309–315.

Furness, S. G., & McNagny, K. (2006). Beyond mere markers: functions for CD34 family of sialomucins in hematopoiesis. *Immunologic Research*, *34*(1), 13–32.

Gabdoulline, R., Kaisers, W., Gaspar, A., Meganathan, K., Doss, M. X., Jagtap, S., Hescheler, J., Sachinidis, A., & Schwender, H. (2015). Differences in the early development of human and mouse embryonic stem cells. *PLoS ONE*, *10*(10), 1–16.

Gaiger, A., Neumeister, A., Heinzl, H., Pabinger, I., & Panzer, S. (1994). HLA class-I and II antigens in chronic idiopathic autoimmune thrombocytopenia. *Annals of Hematology*, *68*(6), 299–302.

Gao, Y., Smith, E., Ker, E., Campbell, P., Cheng, E. chun, Zou, S., Lin, S., Wang, L., Halene, S., & Krause, D. S. (2012). Role of RhoA-Specific Guanine Exchange Factors in Regulation of Endomitosis in Megakaryocytes. *Developmental Cell*, *22*(3), 573–584.

Goodell, M. a, Rosenzweig, M., Kim, H., Marks, D. F., DeMaria, M., Paradis, G., Grupp, S., Sieff, C., Mulligan, R., & CJohnson, R. P. (1997). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. *Nature Medicine*, *3*(12), 1337–1345.

Grozovsky, R., Giannini, S., Falet, H., & Hoffmeister, K. M. (2015). Novel mechanisms of platelet clearance and thrombopoietin regulation. *Current Opinion in Hematology*, *22*(5), 445–451.

Gu, D., Ge, J., Du, W., Xue, F., Chen, Z., Zhao, H., Zhou, Z., Xu, J., Liu, P., Zhao, Q., Zhang, L., & Yang, R. (2009). Raised expression of APRIL in Chinese patients with immune thrombocytopenia and its clinical implications. *Autoimmunity*, *42*(8), 692–698.

Guo, Y., Lubbert, M., & Engelhardt, M. (2003). CD34- hematopoietic stem cells: current concepts and controversies. *Stem Cells*, *21*, 15–20.

Haas, S., Hansson, J., Klimmeck, D., Loeffler, D., Velten, L., Uckelmann, H., Wurzer, S., Prendergast, Á., Schnell, A., Hexel, K., Santarella-Mellwig, R., Blaszkiewicz, S., Kuck, A., Geiger, H., Milsom, M., Steinmetz, L., Schroeder, T., Trumpp, A., Krijgsveld, J., & Essers, M. A. G. (2015). Inflammation-Induced Emergency Megakaryopoiesis Driven by Hematopoietic Stem Cell-like Megakaryocyte Progenitors. *Cell Stem Cell*, *17*(4), 422–434.

Huang, H., Yu, M., Akie, T. E., Moran, T. B., Woo, a. J., Tu, N., Waldon, Z., Lin, Y. Y., Steen, H., & Cantor, a. B. (2009). Differentiation-Dependent Interactions between RUNX-1 and FLI-1 during Megakaryocyte Development. *Molecular and Cellular Biology*, *29*(15), 4103–4115.

Imbach, P., & Crowther, M. (2011). Thrombopoietin-receptor agonists for primary immune thrombocytopenia. *The New England Journal of Medicine*, *365*(8), 734–741.

Ji, L., Zhan, Y., Hua, F., Li, F., Zou, S., Wang, W., Cheng, Y. (2012). The Ratio of Treg/Th17 Cells Correlates with the Disease Activity of Primary Immune Thrombocytopenia. *PLoS ONE*, *7*(12), 1–9.

Ji, X., Zhang, L., Peng, J., & Hou, M. (2014). T cell immune abnormalities in immune thrombocytopenia. *Journal of Hematology & Oncology*, *7*(1), 72.

Keir, M. E., Butte, M. J., Freeman, G. J., & Sharpe, A. H. (2008). PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol*, *26*, 677–704.

Keir, M. E., Latchman, Y. E., Freeman, G. J., & Sharpe, A. H. (2005). Programmed death-1 (PD-1):PD-ligand 1 interactions inhibit TCR-mediated positive selection of thymocytes. *Journal of Immunology (Baltimore, Md. : 1950)*, *175*(11), 7372–9.

Kistangari, G., & McCrae, K. R. (2013). Immune thrombocytopenia. *Hematology/Oncology Clinics of North America*, *27*(3), 497–520.

Kosugi, S., Kurata, Y., Tomiyama, Y., Tahara, T., Kato, T., Tadokoro, S., … Matsuzawa, Y. (1996). Circulating thrombopoietin level in chronic immune thrombocytopenic purpura. *Br J Haematol*, *93*(3), 704–706.

Krause, B. D. S., Fackler, M. J., Civin, C. I., & May, W. S. (1996). CD34: Structure, Biology, and Clinical Utility. *Blood*, *87*(1), 1–13.

Kuipers, H., Muskens, F., Willart, M., Hijdra, D., van Assema, F. B. J., Coyle, A. J.,

Hoogsteden, H., & Lambrecht, B. N. (2006). Contribution of the PD-1 ligands/PD-1 signaling pathway to dendritic cell-mediated CD4+ cell activation. *European Journal of Immunology*, *36*(9), 2472–2482.

Kuvardina, O. N., Herglotz, J., Kolodziej, S., Kohrs, N., Herkt, S., Wojcik, B., Oellerich, T., Corso, J., Behrens, K., Kumar, A., Hussong, H., Urlaub, H., Koch, J., Serve, H., Bonig, H., Stocking, C., Rieger, MA., & Lausen, J. (2015). RUNX1 represses the erythroid gene expression program during megakaryocytic differentiation. *Blood*, *125*(23), 3570–3579.

Kuwana, M., & Ikeda, Y. (2005). The role of autoreactive T-cells in the pathogenesis of idiopathic thrombocytopenic purpura. *International Journal of Hematology*, *81*(2), 106–112.

Levis, M., & Small, D. (2003). FLT3: ITDoes matter in leukemia. *Leukemia : Official Journal of the Leukemia Society of America, Leukemia Research Fund, U.K*, *17*(9), 1738–1752.

Li, J., Xia, Y., & Kuter, D. J. (2000). The platelet thrombopoietin receptor number and function are markedly decreased in patients with essential thrombocythaemia. *British Journal of Haematology*, *111*(3), 943–953.

Li, Z., Yang, F., Dunn, S., Gross, A. K., & Smyth, S. S. (2011). Platelets as immune mediators: Their role in host defense responses and sepsis. *Thrombosis Research*, *127*(3), 184–188.

Marshall, N. B., & Swain, S. L. (2011). Cytotoxic CD4 T cells in antiviral immunity. *Journal of Biomedicine and Biotechnology*, *2011*.

McCrae, K. (2011). Immune thrombocytopenia: No longer 'idiopathic'. *Cleveland Clinic Journal of Medicine*, *78*(6), 358–362.

McMillan, R. (2003). Antiplatelet antibodies in chronic adult immune thrombocytopenic purpura: assays and epitopes. *Journal of Pediatric Hematology/oncology : Official Journal of the American Society of Pediatric Hematology/Oncology*, *25 Suppl 1*(December), S57–S61.

McMillan, R. (2007). The Pathogenesis of Chronic Immune Thrombocytopenic Purpura. *Seminars in Hematology*, *44*(SUPPL. 5).

McMillan, R., Wang, L., & Tani, P. (2003). Prospective evaluation of the immunobead assay for the diagnosis of adult chronic immune thrombocytopenic purpura (ITP). *Journal of Thrombosis and Haemostasis : JTH*, *1*(3), 485–491.

McMillan, R., Wang, L., Tomer, A., Nichol, J., & Pistillo, J. (2004). Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. *Blood*, *103*(4), 1364–1369.

Mcmorran, B. J., Marshall, V. M., Graaf, C. De, Drysdale, K. E., Shabbar, M., Smyth, G. K., Corbin, J., Alexander, W., & Foote, S. J. (2009). Platelets Kill Intraerythrocytic Malarial Parasites and Mediate Survival to Infection. *Platelets*, *323*(February), 797–800.

Moreau, T., Evans, A. L., Vasquez, L., Tijssen, M. R., Yan, Y., Trotter, M. W., Howard, D., Colzani, M., Arumugam, M., Wu, W., Dalby, A., Lampela, R., Bouet, G., Hobbs, C., Pask, D., Payne, H., Ponomaryov, T., Brill, A., Soranzo, N., Ouwehand, W., Pedersen, R., & A Ghevaert, C. (2016). Large-scale production of megakaryocytes from human pluripotent stem cells by chemically defined forward programming. *Nature Communications*, *7*, 112-08.

Mosaad, Y. M. (2014). Hematopoietic stem cells: An overview. *Transfusion and Apheresis Science*, *51*(3), 68–82.

Nakauchi, H., Takano, H., Ema, H., & Osawa, M. (1999). Further characterization of CD34-low/negative mouse hematopoietic stem cells. *Annals of the New York Academy of Sciences*, *872*, 57–70.

Neunert, C., Lim, W., & Crowther, M. (2011). The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. *Blood*, *117*(16), 4190–4207.

Okada, Y., Watanabe, M., Nakai, T., Kamikawa, Y., Shimizu, M., Fukuhara, Y., Yonekura, M., Matsuura, E., Hoshika, Y., Nagai, R., Aird, W., & Doi, T. (2013). RUNX1, but not its familial platelet disorder mutants, synergistically activates PF4 gene expression in combination with ETS family proteins. *Journal of Thrombosis and Haemostasis*, *11*(9), 1742–1750.

Okazaki, T., & Honjo, T. (2007). PD-1 and PD-1 ligands: From discovery to clinical application. *International Immunology*, *19*(7), 813–824.

Olsson, B., Andersson, P. O., M., J., Jacobsson, S., Carlsson, B., Carlsson, L. M., & Wadenvik, H. (2003). T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. *Nat Med*, *9*(9), 1123–4.

Pelanda, R., & Torres, R. M. (2012). Central B-cell tolerance: where selection begins. *Cold Spring Harbor Perspectives in Biology*, *4*(4), 1–15.

Pera, M. F., & Trounson, A. O. (2004). Human embryonic stem cells: prospects for development. *Development (Cambridge, England)*, *131*(22), 5515–25.

Portielje, J. E. A. (2001). Morbidity and mortality in adults with idiopathic thrombocytopenic purpura. *Blood*, *97*(9), 2549–2554.

Prokunina, L., Castillejo-López, C., Oberg, F., Gunnarsson, I., Berg, L., Magnusson, V., & Alarcón-Riquelme, M. E. (2002). A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. *Nature Genetics*, *32*(december), 666–669.

Provan, D., & Newland, A. C. (2015). Current Management of Primary Immune Thrombocytopenia. *Advances in Therapy*, *32*(10), 875–887.

Provan, D., Stasi, R., Newland, A. C., Blanchette, V. S., Bolton-maggs, P., Bussel, J. B., Chong, H., Cines, D., Gernsheimer, T., Godeau, B., Grainger, J., Greer, I., Hunt, B., Imbach, P., Lyons, G., Mcmillan, R., Rodeghiero, F., Sanz, M., Tarantino, M., Watson, S., Young, J., & Kuter, D. J. (2010). Review article International consensus report on the

investigation and management of primary immune thrombocytopenia. *Blood*, *115*(2), 168–187.

Psaila, B., & Bussel, J. B. (2007). Immune Thrombocytopenic Purpura. *Hematology/Oncology Clinics of North America*, *21*(4), 743–759.

Quezada, S. A., Simpson, T. R., Peggs, K. S., Merghoub, T., Vider, J., Fan, X., Blasberg, R., Yagita, H., Muranski, P., Antony, P., Restifo, N., & Allison, J. P. (2010). Tumor-reactive CD4(+) T cells develop cytotoxic activity and eradicate large established melanoma after transfer into lymphopenic hosts. *The Journal of Experimental Medicine*, *207*(3), 637–50.

Ravid, K., Lu, J., Zimmet, J. M., & Jones, M. R. (2002). Roads to polyploidy: The megakaryocyte example. *Journal of Cellular Physiology*, *190*(1), 7–20.

Roark, J. H., Bussel, J. B., Cines, D. B., & Siegel, D. L. (2002). Genetic analysis of autoantibodies in idiopathic thrombocytopenic purpura reveals evidence of clonal expansion and somatic mutation. *Blood*, *100*(4), 1388–1398.

Rodeghiero, F., Stasi, R., Gernsheimer, T., Michel, M., Provan, D., Donald, M., Bussel, J., Cines, D., Chong, H., Cooper, N., Godeau, B., Lechner, K., Mazzucconi, M., Mcmillan, R., Sanz, M., Imbach, P., Kühne, T., Ruggeri, M., George, J., & Arnold, D. M. (2009). Standardization of terminology , definitions and outcome criteria in immune thrombocytopenic purpura of adults and children : report from an international working group Standardization of terminology , definitions and outcome criteria in immune thrombocyt. *Blood*, *113*(11), 2386–2393.

Sanjuan-Pla, A., Macaulay, I. C., Jensen, C. T., Woll, P. S., Luis, T. C., Mead, A., Moore, S., Carella, C., Matsuoka, S., Jones, T., Chowdhury, O., Stenson, L., Lutteropp, M., Green, J., Facchini, R., Boukarabila, H., Grover, A., Gambardella, A., Thongjuea, S., Carrelha, J., Tarrant, P., Atkinson, D., Clark, S A., Nerlov, C., & Jacobsen, S. E. (2013). Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature*, *502*(7470), 232–236.

Sato, T., Laver, J. H., & Ogawa, M. (1999). Reversible Expression of CD34 by Murine Hematopoietic Stem Cells. *Blood*, *94*(8), 2548–2554.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J., White, D., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682.

Schmidt, J., Dojcinovic, D., Guillaume, P., & Luescher, I. (2013). Analysis, isolation, and activation of antigen-specific CD4+ and CD8+ T cells by soluble MHC-peptide complexes. *Frontiers in Immunology*, *4*(JUL), 1–14.

Schoonen, W. M., Kucera, G., Coalson, J., Li, L., Rutstein, M., Mowat, F., Kaye, J. A. (2009). Epidemiology of immune thrombocytopenic purpura in the General Practice Research Database. *Br J Haematol*, *145*(2), 235–244.

Semple, J. W. (2013). The cellular pathophysiology of immune thrombocytopenia. *ISBT Science Series*, *8*(1), 210–213.

Semple, J. W., & Freedman, J. (1995). Abnormal cellular immune mechanisms associated with autoimmune thrombocytopenia. *Transfusion Medicine Reviews*, *9*(4), 327–338.

Semple, J. W., Italiano, J. E., & Freedman, J. (2011). Platelets and the immune continuum. *Nature Reviews Immunology*, *11*(4), 264–74.

Shao, L., Wu, Y., Zhou, H., Qin, P., Ni, H., Peng, J., & Hou, M. (2014). Successful treatment with oseltamivir phosphate in a patient with chronic immune thrombocytopenia positive for anti-GPIb/IX autoantibody. *Platelets*, *00*(00), 1–3.

Shrimp, J. H., Hu, J., Dong, M., Wang, B. S., Macdonald, R., Jiang, H., Hao, Q., Yen, A., & Lin, H. (2014). Revealing CD38 cellular localization using a cell permeable, mechanism-based fluorescent small-molecule probe. *Journal of the American Chemical Society*, *136*(15), 5656–5663.

Spurr, E. E., Wiggins, N. E., Marsden, K. A., Lowenthal, R. M., & Ragg, S. J. (2002). Cryopreserved human haematopoietic stem cells retain engraftment potential after extended (5-14 years) cryostorage. *Cryobiology*, *44*(3), 210–217.

Stasi, R., Cooper, N., Poeta, G. Del, Stipa, E., Evangelista, M. L., Abruzzese, E., & Amadori, S. (2008). Brief report Analysis of regulatory T-cell changes in patients with idiopathic thrombocytopenic purpura receiving B cell – depleting therapy with rituximab, *112*(4), 1147–1150.

Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A., & Sharpe, A. H. (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity*, *3*(5), 541–547.

Toltl, L., Nazi, I., Jafari, R., & Arnold, D. (2011). Piecing Together the Humoral and Cellular Mechanisms of Immune Thrombocytopenia. *Seminars in Thrombosis and Hemostasis*, *37*(06), 631–639.

Tomer, A. (2004). Human marrow megakaryocyte differentiation: Multiparameter correlative analysis identifies von Willebrand factor as a sensitive and distinctive marker for early (2N and 4N) megakaryocytes. *Blood*, *104*(9), 2722–2727.

Tomer, A., & Harker, L. A. (1996). Measurements of In Vivo Megakaryocytopoiesis: Studies in Nonhuman Primates and Patients. *Stem Cells*, *14*(1), 18–30.

Trinh, B. Q., Barengo, N., Kim, S. B., Lee, J.-S., Zweidler-McKay, P. A., & Naora, H. (2015). The homeobox gene DLX4 regulates erythro-megakaryocytic differentiation by stimulating IL-1 and NF- B signaling. *Journal of Cell Science*, *128*(16), 3055–3067.

van Dijk-Hard, I., Feld, S., Holmberg, D., & Lundkvist, I. (1999). Increased utilization of the VH6 gene family in patients with autoimmune idiopathic thrombocytopenic purpura. *Journal of Autoimmunity*, *12*(1), 57–63.

Wang, L., Pino-Lagos, K., de Vries, V. C., Guleria, I., Sayegh, M. H., & Noelle, R. J.

(2008). Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(27), 9331–6.

Wang, S., Bajorath, J., Flies, D. B., Dong, H., Honjo, T., & Chen, L. (2003). Molecular modeling and functional mapping of B7-H1 and B7-DC uncouple costimulatory function from PD-1 interaction. *The Journal of Experimental Medicine*, *197*(9), 1083–1091.

Woolthuis, C. M., & Park, C. Y. (2016). Hematopoietic stem/progenitor cell commitment to the megakaryocyte lineage. *Blood*, *127*(10), 1242–1249.

Yang, L., Bryder, D., Adolfsson, J., Nygren, J., Månsson, R., Sigvardsson, M., & Jacobsen, S. E. W. (2005). Identification of Lin_-Sca1+kit+CD34+Flt3_- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood*, *105*(7), 2717–2723.

Ye, X., Zhang, L., Wang, H., Chen, Y., Zhang, W., & Zhu, R. (2015). The Role of IL-23 / Th17 Pathway in Patients with Primary Immune Thrombocytopenia. *PLoS ONE*, *10*(1), 1–13.

Zhu, F., Qiao, J., Cao, J., Sun, H. Y., Wu, Q. Y., Sun, Z. T., Zhao, Kai., Sang, W., Qi, K., Zeng, L., Li, Z., & Xu, K. L. (2015). Decreased level of cytotoxic T lymphocyte antigen-4 (CTLA-4) in patients with acute immune thrombocytopenia (ITP). *Thrombosis Research*, *136*(4), 797–802.

Zwicker, J. I., Lacroix, R., Dignat-george, F., Furie, B. C., & Furie, B. (2012). Platelets and Megakaryocytes. *Methods*, *788*, 127–139.